# Characterization of the protease phenotype of cultured human mast cells during their differentiation and maturation

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**MASTER'S THESIS**


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## ABSTRACT


**Background and Aims:** Mast cells (MCs) are tissue-dwelling effector cells of innate and adaptive immunity that differentiate in peripheral tissues from committed circulating progenitor cells of bone marrow origin. Human MCs are conventionally classified into two major subtypes based on their neutral protease content, namely the $MC_T$, which contain only tryptase and the $MC_{TC,}$ which contain both tryptase and chymase, as well as carboxypeptidase A3 and cathepsin G. Granzyme B has been identified in cultured human MCs and in MCs of human skin. *In vitro* studies have helped trace factors that regulate human MC development and have provided a powerful method for studying neutral protease expression during MC development. However, inconsistent information from these studies has made it impossible to establish a consistent concept of what causes MCs to develop into $MC_T$ and $MC_{TC}$: are they two committed subtypes deriving from two distinct progenitors with irreversibly predetermined protease phenotype, or are they functional states that MCs assume under the influence of the local microenvironment? Previous findings of human tissue MCs further imply that the phenotypic heterogeneity of human MCs may be greater than initially suggested. However, an understanding of the complexity of the phenotypic heterogeneity of human MCs, and to what extent this heterogeneity is intrinsically variable, has been hindered. This is because virtually all studies describing the relationship of human MC development and protease expression have focused on the expression of tryptase and/or chymase, whereas the expressions of carboxypeptidase A3, cathepsin G, and granzyme B by human MCs have not been fully considered. The aim of the present Master's thesis was to clarify the contradictions on human MC phenotypes and their development by studying how MCs derived from their circulating progenitors express the various neutral proteases during their development.

**Methods:** MCs were generated from human peripheral blood-derived $CD34^+$ progenitors under the influence of Kit ligand and sequentially added cytokines according to a previously published method. The protease expressions of tryptase, chymase, carboxypeptidase A3, cathepsin G, and granzyme B were investigated on a weekly basis during MC development by flow cytometry and quantitative PCR. Furthermore, immunostainings of the proteases were performed for immunofluorescence microscopy.

**Results:** All investigated proteases were detected in the developing MCs at week 1 of culture and were increasingly expressed beyond that time. By the end of week 6, a single homogeneous population of cells expressing all the investigated proteases was observed.

**Conclusion:** The data of the present study suggest that human MCs derive from a common circulating progenitor cell, which has the potential to express the full complement of the investigated proteases. Thus, the heterogeneity of human MC protease phenotypes reflects microenvironmental regulation of protease expression, rather than the existence of distinct progenitor cells with precommitted protease phenotypes.

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## TIIVISTELMÄ


**Tausta ja Tavoitteet:** Syöttösolut ovat ihmisen synnynnäisen ja hankitun immuniteetin keskeisiä toimijasoluja, jotka erilaistuvat kudoksissa luuytimestä peräisin olevista, verenkierrossa kiertävistä esiastesoluista. Ihmisen syöttösolut jaetaan perinteisesti kahteen alatyyppiin niiden sisältämien neutraaliproteaasien perusteella. Nämä ovat $MC_T$ syöttösolu, joka sisältää ainoastaan tryptaasia ja $MC_{TC}$ syöttösolu, joka sisältää tryptaasia ja kymaasia. Jälkimmäiseen alatyyppiin kuuluvat syöttösolut sisältävät myös karboksipeptidaasi A3:a ja katepsiini G:tä. Lisäksi ihmisen viljellyt ja ihosta eristetyt syöttösolut ilmentävät grantsyymi B:tä. Ihmisen syöttösolujen erilaistaminen viljelyolosuhteissa on mahdollistanut neutraaliproteaasien ilmentymisen tutkimisen syöttösolujen erilaistumisen ja kasvun aikana. Tästä huolimatta on epäselvää, ovatko $MC_T$ ja $MC_{TC}$ peräisin kahdesta eri kehityslinjasta vai ovatko ne ennemminkin syöttösolun toiminnallisia tiloja, jotka määräytyvät ympärillä vallitsevien olosuhteiden mukaan. Viime vuosien löydökset viittaavat lisäksi siihen, että ihmisen syöttösolujen proteaasi-ilmiasujen kirjo on luultua monimuotoisempaa. Se, kuinka kirjavaa tämä monimuotoisuus on, ja missä määrin se on synnynnäisesti vaihtelevaa, on kuitenkin epäselvää. Tämä johtuu pääosin siitä, että valtaosa tutkimuksista on keskittynyt vain tryptaasin ja/tai kymaasin ilmentymiseen, kun taas muiden neutraaliproteaasien ilmentymistä ei ole juurikaan tutkittu. Tässä tutkimuksessa seurattiin kaikkien tunnettujen ihmisen syöttösoluproteaasien ilmentymistä viljeltyjen syöttösolujen erilaistumisen ja kasvun aikana. Tavoitteena oli selventää nykyistä käsitystä ihmisen syöttösolujen proteaasi-ilmiasujen monimuotoisuudesta ja niistä tekijöistä, jotka ovat tämän monimuotoisuuden takana.

**Menetelmät:** Syöttösolut erilaistettiin viljelemällä verenkierrosta eristettyjä $CD34^+$ esiastesoluja tärkeimmän syöttösolujen kasvua edistävän kasvutekijän, Kit ligandin ja jaksoittain lisättyjen sytokiinien, interleukiini (IL)-3:n, IL-9:n ja IL-6:n kanssa. Solujen erilaistumisen ja kasvun aikana tryptaasin, kymaasin, karboksipeptidaasi A3:n, katepsiini G:n ja grantsyymi B:n ilmentymistä tutkittiin viikoittain kvantitatiivisella PCR:lla ja virtaussytometrialla. Lisäksi tehtiin immunovärjäyksiä mikroskopointia varten.

**Tulokset:** Kaikki tutkitut proteaasit tunnistettiin syöttösolujen kehittymisen aikana sekä mRNA että proteiinitasolla jo ensimmäisen viljelyviikon jälkeen. Proteaasien ilmentymistasot nousivat viljelyn edetessä, ja kuuden viikon jälkeen kaikki syöttösolut ilmensivät tutkittuja proteaaseja. Yhdenkään proteaasin kohdalla ei havaittu erillisiä proteaasipositiivisia tai proteaasinegatiivisia alapopulaatioita, vaan jokaisessa tutkitussa aikapisteessä havaittiin yksi syöttösolupopulaatio, joka ilmensi vaihtelevalla tasolla tutkittua proteaasia.

**Yhteenveto:** Tämän tutkimuksen tulokset viittaavat siihen, että ihmisen syöttösolut ovat peräsin yhteisestä esiastesolusta, joka pystyy ilmentämään kaikkia tutkittuja neutraaliporteaaseja. Syöttösolujen proteaasi-ilmiasujen heterogeenisyys on seurausta paikallisessa kudosympäristössä olevista tekijöistä, kuten sytokiineista, joilla on kyky säädellä proteaasien ilmentymistasoja.

Abbreviations

| | |
|---|---|
| APC | Allophycocyanin |
| BMMC | Bone marrow-derived mast cell |
| BSA | Bovine serum albumin |
| c-KIT/KIT | Receptor for stem cell factor/kit ligand |
| CPA3 | Carboxypeptidase A3 |
| CTMC | Connective tissue mast cell |
| ECM | Extracellular matrix |
| FcεRI/IgERI | High affinity receptor for immunoglobulin E |
| FITC | Fluorescein isothiocyanate |
| GAG | Glycosaminoglycan |
| HDC | Histidine decarboxylase |
| hLDL | Human low-density lipoprotein |
| IFN-γ | Interferon-γ |
| IgG | Immunoglobulin G |
| IgE | Immunoglobulin E |
| IL | Interleukin |
| KITLG | Kit ligand |
| IMDM | Iscove's Modified Dulbecco's Medium |
| mAb | Monoclonal antibody |
| MC | Mast cell |
| $MC_C$ | Mast cell containing only chymase |
| $MC_T$ | Mast cell containing only tryptase |
| $MC_{TC}$ | Mast cell containing both tryptase and chymase |
| MFI | Mean fluorescence intensity |
| MMC | Mucosal mast cell |
| mMCP | Mouse mast cell protease |
| NGF | Nerve growth factor |
| pAb | Polyclonal antibody |
| PBMC | Peripheral blood mononuclear cell |
| PE | Phycoerythrin |
| qRT-PCR | Quantitative real-time reverse-transcription PCR |
| rMCP | Rat mast cell protease |
| SCF | Stem cell factor |
| TGF-β | Transforming growth factor-β |

CONTENTS

# 1. INTRODUCTION

Mast cells (MCs) are tissue-dwelling multifunctional effector cells best known for their pivotal role in IgE-mediated allergic disorders (Galli & Tsai, 2012). The hallmark of tissue MCs is their numerous cytoplasmic secretory granules, which store a wide variety of biologically active mediators, most importantly histamine, heparin proteoglycan, and various neutral proteases (Metcalfe et al., 1997). By releasing these, and also other mediators, such as various cytokines, chemokines and lipid mediators upon activation, MCs can elicit various anti-inflammatory and pro-inflammatory functions in the body (Galli et al., 2008). MCs have been identified in all vertebrate classes, however, MCs in different species, as well as at distinct anatomical sites display marked plasticity in their morphological, histochemical, biochemical, and functional characteristics and phenotypically distinct subtypes of MCs can be found within the tissues of humans and other species (Huff & Lanz, 1997; Metcalfe et al., 1997). Human MCs are conventionally classified into two distinct subtypes based on their neutral protease composition, the $MC_T$ containing exclusively tryptase and the $MC_{TC}$ containing both tryptase and chymase, as well as carboxypeptidase A3 and cathepsin G (Irani et al., 1986; Irani et al., 1989; Schechter et al., 1990; Irani et al., 1991). Cultured human MCs and MCs of human skin also contain granzyme B (Strik et al., 2007).

Human MCs derive from committed circulating progenitor cells of bone marrow origin and differentiate in peripheral tissues under the influence of the local microenvironment (Okayama & Kawakami, 2006). MCs are present in virtually all vascularized tissues, however, quite different proportions of $MC_{TC}$ to $MC_T$ have been reported within various tissues (Weidner & Austen, 1993; Irani & Schwartz, 1994), and specific functions for $MC_{TC}$ and $MC_T$ have been suggested (McNeil & Gotis-Graham, 2000). *In vitro* studies have provided a powerful tool for the investigation of the relationship between MC development and protease expression however, the developmental pathway leading to $MC_T$ and $MC_{TC}$ has remained a subject of conflicting evidence. Furthermore, previous findings of MCs within human tissues imply that the phenotypic diversity of the human MC is greater than initially suggested (Weidner & Austen, 1993; Abonia et al., 2010; Dougherty et al., 2010).

This Master's thesis is based on a published piece of work, which provided the first study to investigate the expression of various human MC neutral proteases, namely tryptase, chymase, CPA3, cathepsin G, and granzyme B during the development of human MCs from their progenitors present in the adult circulating blood (Maaninka et al., 2013).

2. REVIEW OF THE LITERATURE

## 2.1. Mast cells —Historical perspective

The German pathologist Friedrich von Recklinghausen first described MCs cells in 1863 when he found these granular cells in the mesentery of the frog. However, Paul Ehrlich, the German scientist, who received the Nobel Prize for his contributions to immunology, is credited with the discovery of MCs, as we know them today. Ehrlich discovered MCs in 1877, while he was still a medical student at Freiburg University. The discovery was based on the specific staining characteristics of MC cytoplasmic granules. Ehrlich noticed that when connective tissues were stained with aniline blue, certain cells dyed red. After precise dissection, he noticed that the stained cells were full of cytoplasmic granules that had turned from a blue to a reddish color, a phenomenon referred to as metachromasia (Fig. 1). Ehrlich believed that the MC granules were the result of overfeeding, and named the cells Mastzellen based on "mästen" in German, which refers to feeding (Schwartz & Huff, 1998). In addition to MCs, which he found associated with blood vessels, inflamed tissues, and nerves, Ehrlich described basophils as metachromatic cells that circulated in the blood. Based on his pioneering studies on blood cell polychromatophilia, which led to the systematic and fundamental classification of blood cells still in clinical use, Ehrlich is generally considered the founder of hematology.

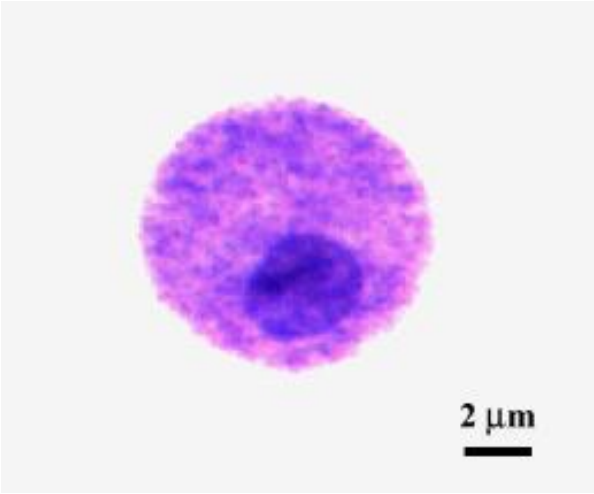


**Figure 1. Cultured human MC displaying metachromatic staining.** Human MCs were cultured for 9 weeks and stained with toluidine blue (Lappalainen et al., 2007).

Since the initial description of Ehrlich, MCs have been identified by their metachromatic staining properties. The metachromasia exhibit by MCs is based on interaction of basic dyes such as Alcian blue with the highly sulfated glycosaminoglycans (GAG), notably heparin, within MC secretory granules (Metcalfe et al., 1997). The presence of heparin within MCs was initially described in the 1930 and was confirmed in the 1960s by a study showing that heparin is a main granule component of rat serosal MCs (Lagunoff et al., 1964; Rönnberg et al., 2012). Soon after that Enerbäck, however, noticed that tissue MCs in rats exhibit differences in fixation and dye-binding properties, an observation that provided the first evidence of MC heterogeneity (Enerbäck, 1966). Heterogeneity between tissue MCs was confirmed and extended by immunohistochemcial studies of Woodbury and Miller by showing that the MCs present in the peritoneal cavity of rat differed from those in the jejunum in the terms of their expression of the chymase-like serine proteases they designated as rat mast cell protease (rMCP)-1 and rMCP-2, respectively (Woodbury et al., 1978; Gibson & Miller, 1986). In support of these histochemistry and protease data, it was shown that the two populations of rat MCs, designated as connetive tissue MCs (CTMC) and mucosal MCs (MMC) stored serglycin proteoglycans in their secretory granules that contained different GAGs (Metcalfe et al., 1997). This finding explained the differences in histochemical staining properties initially described by Enerbäck by showing that the differences in fixation and dye-binding properties of rodent MC subtypes is causally related to the presence of heparin only in the CTMC, whereas both subtypes contain chondroitin sulfates (Huff & Lanz, 1997).

By analogy to rodents, two major MC subtypes have been described within human tissues. In contrast to rodents, all mature human MCs contain heparin, and thus heparin-dependent fixation and histochemical procedures used in rodents cannot be used to distinguish human MCs (Metcalfe et al., 1997). Instead, human MCs are divided into two distinct subtypes based on their neutral protease composition. The concept of the existence within human tissues of two distinct MC subtypes distinguishable by their neutral protease composition is based on immunohistochemical studies of Schwartz and his group in the 1980s. By using newly developed antibodies against tryptase and chymase, Schwartz and coworkers found that certain MCs stained positive for tryptase only, whereas others stained positive for both tryptase and chymase. Based on the absence or presence of chymase these MCs were named $MC_T$ and $MC_{TC}$ to indicate an MC containing tryptase only and a MC containing both tryptase and chymase respectively (Irani et al., 1986; Irani et al., 1989). However, this nomenclature is not optimal because further studies demonstrated that $MC_{TC}$ also contain the neutral proteases carboxypeptidase A3 (CPA3) and cathepsin G (Schechter et al., 1990; Irani et al., 1991). Besides $MC_T$ and $MC_{TC}$, two additional human MC phenotypes, namely $MC_C$

indicating an MC containing chymase only and an MC containing tryptase and CPA3 but not chymase have been identified within human tissues (Weidner & Austen, 1993; Abonia et al., 2010; Dougherty et al., 2010). However, the existence of $MC_C$ has remained a subject of conflicting evidence, since many authors have failed to detect it (Irani et al., 1989; Tetlow & Woolley, 1995; Gotis-Graham & McNeil, 1997). Furthermore, an MC containing tryptase and CPA3 has been relatively recently identified (in 2010) within human tissues (Abonia et al., 2010; Dougherty et al., 2010), and thus its presence has not been fully established.

### 2.2. Mast cell neutral proteases

Neutral proteases are proteolytic enzymes having a neutral-to-slightly-basic pH optimum, and are the main protein constituents of the secretory granules of human MCs. Apart from one, the CPA3, which is a zinc-containing exopeptidase of the metalloproteinase family, all MC neutral proteases are serine proteases characterized by an active site serine residue.

There are remarkable variations in the composition of neutral proteases within the MCs between different species, especially between human and rodent species. Neutral protease activity was first described in the MCs of humans, dogs, rats, mice, and rabbits in the early 1950s by a histochemical technique involving cleavage of the chromogenic substrate 3-chloroacetoxy-2-napthoic acid anilide (Gomori, 1953). A couple of years later, this activity was characterized as chymotrypsin-like because it hydrolyzed acetyl ethyl esters of aromatic but not basic amino acids (Benditt & Arase, 1959). In the 1960s, using other substrate-based techniques, human skin MCs were reported to contain substantial levels of trypsin-like enzyme activity. However, it was a couple of decades until tryptase and chymase responsible for trypsin-like and chymotrypsin-like enzyme activities, respectively, were first isolated and characterized (Schwartz et al., 1981; Schechter et al., 1983). To date at least six MC-specific neutral proteases, namely four tryptases (α-, β-, γ-, and δ-tryptases), chymase, and CPA3, as well as two additional serine proteases, cathepsin G and granzyme B, which are classically related to neutrophils, and to cytotoxic T-cells and natural killer cells, respectively, have been identified in human MCs (Schechter et al., 1983; Goldstein et al., 1987; Schechter et al., 1990; Caughey, 2007; Strik et al., 2007). A novel human MC protease, granzyme H has been reported in human MCs, but it was characterized after the experimental part of this Thesis was completed (Rönnberg et al., 2014).

### *2.2.1. Human mast cell neutral proteases*

#### *2.2.1.1. Tryptase*

Tryptase is present in most, if not all, human MCs (Metcalfe et al., 1997). Tryptase has trypsin-like cleavage specificity, as it cleaves protein substrates at the C-terminal side of arginine and lysine residues (Schwartz et al., 1981; Tanaka et al., 1983). However, unlike the many tryptic peptidases associated with digestion, hemostasis, clot lysis, and complement activation, tryptase is highly selective regarding its peptide and protein targets (Schwartz et al., 1981; Tanaka et al., 1983), which underscores its unique position in the hierarchy of peptidases belonging to the trypsin family. MC tryptase has several unique features, one of the most remarkable ones being its organization into a tetrameric state with the active sites oriented toward a narrow central pore, and its consequent resistance to endogenous macromolecular protease inhibitors, such as serpins and α2-macroglobulin (Caughey, 1997).

The human MC tryptase locus resides on chromosome 16p13.3 and spans approximately 1.6 Mb (Pallaoro et al., 1999). To date, four MC tryptase genes (TPSAB1, TPSB2, TPSD1, and TPSG1) plus various pseudogenes have been identified in humans (Pallaoro et al., 1999; Caughey et al., 2000). These fall into two major groups: the soluble α-, β-, and δ-tryptases and the membrane-anchored γ-tryptase, also known as transmembrane tryptase or TMT (Pallaoro et al., 1999). Of these, the β-tryptase appears to be the main form stored in human MC granules, and it occurs in three almost identical forms: βI, βII, and βIII (Miller et al., 1990; Vanderslice et al., 1990).

Among the α-tryptases, two very similar forms have been identified in humans: αI and αII (Miller et al., 1989; Pallaoro et al., 1999). However, the activity of human α-tryptase is extremely low compared with β-tryptase, which is partly due to the amino acid substitution of glycine for asparagine at the position 216 of the substrate-binding pocket (Huang et al., 1999). It has been suggested that, in contrast to β-tryptase, which is stored in the secretory granules and not released unless the MCs have been challenged by a degranulating stimulus, α-tryptase is constitutively released via a selective pathway (Schwartz et al., 1995). From δ-tryptase, two nearly identical forms (δI and δII) have been identified (Wang et al., 2002). However, the activity of δ-tryptase is also much lower than that of β-tryptase, which is mainly due to a premature stop codon that results in a truncated protein and affects the substrate specificity of δ-tryptase significantly (Wang et al., 2002).

Finally, two different forms of the human transmembrane tryptase (γ-tryptase) have been identified: γI and γII (Caughey et al., 2000). The γ-tryptases contain an extended hydrophobic C-terminal domain followed by a small cytoplasmic tail, are anchored in either the plasma membrane or the secretory granule membrane, and only act locally upon MC activation (Caughey et al., 2000).

#### *2.2.1.2. Chymase*

In humans, the major chymotryptic protease, as defined by its preference for cleaving peptide and protein substrates at the C-terminal site of aromatic amino acids, such as phenylalanine, tyrosine, and tryptophan, is the neutral serine protease chymase (Powers et al., 1985; Caughey, 2007). Only one chymase gene (*CMA1*) has been found in humans (Caughey et al., 1993), and human MCs are conventionally divided into two distinct subtypes, the $MC_{TC}$ and $MC_T$, based on the presence or absence, respectively, of this chymase within their granules. Human MC chymase is located on chromosome 14q11.2 (Caughey et al., 1993) at the end of a small cluster of four genes covering approximately 130 kb. This cluster also contains the cathepsin G gene (*CTSG*) and the granzyme H and B genes (*GZMH* and *GZMB* respectively) (Caughey et al., 1993). The human chymase is active in its monomeric form (Pejler et al., 2007) and has more destructive potential than tryptase, given that it can cleave a fairly wide variety of peptide and protein targets (Caughey, 2007). Befitting its greater destructive potential, chymase is more susceptible to inhibition by circulating and extravascular anti-peptidases, including serpins and α2-macroglobilin, and is thus quickly inhibited after release, although some protection against inhibition is gained by tight binding to co-released proteoglycans, such as heparin (Lindstedt et al., 2001; Caughey, 2007).

#### *2.2.1.3. Carboxypeptidase A3*

Human CPA3 is a zinc-dependent metalloexoprotease that belongs to the carboxypeptidase (CP) A/B family. As indicated by the letter "A" in its name, CPA3 has a CPA-like cleavage specificity, i.e., it prefers cleaving peptide and ester bonds at the amino side of the C-terminal aromatic amino acids (Goldstein et al., 1989). Human CPA3 is encoded by a single gene (*CPA3*), which situates on chromosome 3q24 (indicated by number 3 in its name) and spans over 32 kb (Pejler et al., 2007). The expression of CPA3 is suggested to be MC-specific (Li et al., 1998) and it has been identified in the $MC_{TC}$ and in MCs containing tryptase and CPA3 but not chymase (Goldstein et al., 1989; Abonia et al., 2010; Dougherty et al., 2010). Despite having a CPA-like substrate-binding pocket and enzyme activity, CPA3 is structurally similar to bovine and human pancreatic CPB, which indicates its uniqueness among CPs (Goldstein et al., 1989; Reynolds et al., 1992).

#### *2.2.1.4. Cathepsin G*

Human cathepsin G is a serine protease that belongs to the cathepsin class of enzymes. Unlike tryptase, chymase, and CPA3, which are considered MC-specific proteases, expression of cathepsin G is classically related to neutrophils (Korkmaz et al., 2008). In human MCs, the expression of cathepsin G is mainly restricted to the $MC_{TC}$ type (Schechter et al., 1990). Only one cathepsin G gene (*CTSG*) has been identified in humans, and it is located within a cluster of four genes on chromosome 14q11.2 (Caughey et al., 1993). Similar to chymase, cathepsin G has chymotrypsin-like activity, but cathepsin G is generally a weaker enzyme than chymase in terms of its destructive potential, and it has a broader peptidase specificity; that is, it exhibits the unusual property of having both chymotryptic and tryptic activity (Caughey, 2007).

#### *2.2.1.5. Granzyme B*

The presence of granzyme B, a member of a family of death-inducing serine proteases classically related to granule components of cytotoxic T lymphocytes and natural killer cells (Lieberman, 2003), was identified within human MCs in 2007 (Strik et al., 2007). It was reported to be present in cultured human MCs and MCs of human skin. Five granzyme subtypes have been identified in humans (A, B, H, K, and M) (Barry & Bleackley, 2002), and granzyme B is the most characterized of all (Ngan et al., 2009). Granzyme B is a caspase-like serine protease that cleaves substrates at the carboxyl side of acidic residues, particularly aspartic acid (Fan & Zhang, 2005). The granzyme B of lymphocytes and NK cells is a pro-apoptotic intracellular protease, which requires perforin to form pores in the plasma membrane of the target cells to help its entry into intracellular compartments (Fan & Zhang, 2005). Unlike lymphocytes, MCs do not store perforin in their secretory granules (Pardo et al., 2007), which suggests a unique function of MC-derived granzyme B. However, the extracellular effects of this enzyme are not well understood (Froelich et al., 2009).

### *2.2.2. Rat and mouse mast cell neutral proteases*

Most of our knowledge about the biology of MC neutral proteases is based on studies using experimental animals, most notably rat and mouse. However, there appear to be remarkable differences in the neutral proteases present within MCs of human and rodent species, especially mice, which can store a substantially larger number of different proteases than humans. In contrast to humans, who express a single chymase, several chymase forms can be found within the MCs of

**Table 1. Human, mouse, and rat MC proteases**

| MC Protease | Preferential subtype expressed | Homologues |
|---|---|---|
| ***MC chymases*** | | |
| hChymase 1 | $MC_{TC}$, $MC_{C}$ | mMCP-5, rMCP-5 |
| mMCP-1 | MMC | rMCP-4 |
| mMCP-2 | MMC | rMCP-2 |
| mMCP-4 | CTMC | rMCP-1 |
| mMCP-5 | CTMC | rMCP-5 |
| mMCP-8 | Data not available | rMCP-8, rMCP-9, rMCP-10, |
| mMCP-9 | CTMC | rMCP-3 |
| rMCP-1 | CTMC | mMCP-4 |
| rMCP-2 | MMC | mMCP-2 |
| rMCP-3 | MMC | mMCP-9 |
| rMCP-4 | MMC | mMCP-1 |
| rMCP-5 | CTMC | mMCP-5 |
| rMCP-8 | MMC | mMCP-8 |
| rMCP-9 | MMC | |
| rMCP-10 | MMC | |
| ***MC tryptases*** | | |
| hTryptase αI/βI | $MC_{T}$, $MC_{TC}$, MC containing tryptase and CPA3 | mMCP-6, rMCP-6 |
| hTryptase βII/βIII | $MC_{T}$, $MC_{TC}$, MC containing tryptase and CPA3 | mMCP-6, rMCP-6 |
| hTryptase δ | $MC_{T}$, $MC_{TC}$, MC containing tryptase and CPA3 | mMCP-7, rMCP-7 |
| hTMT/Tryptase γ | $MC_{T}$, $MC_{TC}$, MC containing tryptase and CPA3 | mTMT, rTMT |
| rTMT | CTMC | mTMT, hTMT/Tryptase γ |
| rMCP-6 | CTMC | mMCP-6, hTryptase αI/ βI, hTryptase βII/βIII |
| rMCP-7 | CTMC | mMCP-7, hTryptase δ |
| rMCP-11 | Data not available | mMCP-11 |
| mTMT | Data not available | rTMT, hTMT/Tryptase γ |
| mMCP-6 | CTMC | rMCP-6, hTryptase αI/ βI, hTryptase βII/βIII |
| mMCP-7 | CTMC | rMCP-7, hTryptase δ |
| mMCP-11 | Data not available | rMCP-11 |
| ***CPA*** | | |
| hCPA | $MC_{TC}$, MC containing tryptase and CPA3 | rCPA, mCPA |
| rCPA | CTMC | mCPA, hCPA |
| mCPA | CTMC | rCPA, hCPA |
| ***Cathepsin G*** | | |
| hCathepsin G | $MC_{TC}$ | |
| mCathepsin G | Data not available | |
| ***Granzymes*** | | |
| hGranzyme B | Cultured human MCs, MCs of human skin | |
| hGranzyme H | Cultured human MCs, MCs of human skin | |
| mGranzyme B | Data not available | |
| mGranzyme D | BMMC, PCMC | |
| ***Neuropsin*** | | |
| mNeuropsin | BMMC | |

Data from (Weidner & Austen, 1993; Lunderius & Hellman, 2001; Pardo et al., 2007; Pejler et al., 2007; Stevens & Adachi, 2007; Strik et al., 2007; Abonia et al., 2010; Dougherty et al., 2010; Rönnberg et al., 2013). BMMC; Bone marrow-derived MC, PCMC; Peritoneal cell-derived MC

rats and mice. Furthermore, tryptase expression in rodents appears to be restricted to MC population residing at connective tissues, whereas in humans, tryptase (at least β-tryptase) is found in most, if not all, MCs irrespective of their tissue localization. Current nomenclature decribes MC chymases and tryptases in rat and mouse as rat mast cell proteases (rMCPs) and mouse mast cell proteases (mMCPs), respectively. Table 1 summarizes neutral protease expression in humans, rats, and mice.

### 2.3. Tissue distribution and function of human mast cell subtypes

Quite different proportions of $MC_T$ and $MC_{TC}$ subpopulations have been reported in various human tissues by different groups of investigators. However, there appear to be some rules governing the tissue distribution of the MC subtypes. In histologically normal human tissues, $MC_T$ is the primary subtype at the mucosal surfaces of the respiratory and gastrointestinal tracts, such as in the lung, particularly the alveoli, and the small intestinal mucosa, whereas $MC_{TC}$ cells are the predominant subtype found in the skin, synovium, and gastrointestinal submucosa (Irani et al., 1986; Irani et al., 1989). Although a particular MC subtype appears to predominate in a particular tissue, a fraction of the other subtype is also usually present (Table 2), and the relative abundances of $MC_T$ and $MC_{TC}$ subtypes may change with inflammation and other disease processes (Table 3). Accordingly, MC subtype designation based exclusively on tissue location is not justified.

The localization of MCs at sites close to the external milieu reflects their role as important sentinels of the body. MCs can contribute to many processes of both innate and adaptive immunity and can have both protective and pathogenic roles (Galli & Tsai, 2010). MCs mediate their effector functions by releasing the various neutral proteases and other mediators upon activation by an appropriate stimulus, such as the classical allergic activation caused by crosslinking of receptor-bound IgE by an IgE-specific antigen or allergen (Metcalfe et al., 1997). The process of protease exocytosis that results from the FcεRI signaling pathway is called degranulation (Fig. 2; *right panel*).

Several studies have begun to address the presence of distinct MC subtypes in various human diseases. For example, MC hyperplasia has been noted in rheumatoid synovium at sites of cartilage erosion (Bromley et al., 1984; Tetlow & Woolley, 1995). In the rheumatoid joint, $MC_{TC}$ appear to be associated with areas of dense fibrosis, whereas variable ratios of both $MC_T$ and $MC_{TC}$ cells have

**Table 2. Distribution of $MC_T$, $MC_{TC}$, and $MC_C$ in normal human tissues**

| Tissue | % $MC_T$ | % $MC_{TC}$ | % $MC_C$ |
|---|---|---|---|
| **Skin** | <1 | >99 | |
| **Lung** | | | |
| *Alveoli* | 91/93 | 8/7 | 1/- |
| *Bronchi* | 78 | 10 | 12 |
| *Bronchial epithelium* | 100 | 0 | - |
| *Bronchial subepithelium* | 75 | 25 | - |
| **Axillary lymph nodes** | 1 | 97 | 2 |
| **Breast parenchyma** | 1 | 99 | 0 |
| **Stomach** | | | |
| *Mucosa* | 52 | 39 | 9 |
| *Submucosa* | 0 | 73 | 27 |
| **Small intestine** | | | |
| *Mucosa* | 65/81 | 31/19 | 4/- |
| *Submucosa* | 0/23 | 76/77 | 24/- |
| **Colon** | | | |
| *Mucosa* | 53 | 37 | 10 |
| *Submucosa* | 0 | 96 | 4 |
| **Nasal mucosa** | 66 | 34 | - |
| **Conjunctiva** | 5 | 95 | - |
| **Synovium** | 17/34 | 83/66 | -/- |
| **Heart** | 10 | 90 | - |
| **Kidney** | 65 | 35 | - |
| **Uterus** | | | |
| *Endometrium* | 84 | 16 | - |
| *Inner myometrium* | 48 | 52 | - |
| *Outer myometrium* | 10 | 90 | - |
| *Cervix* | 40 | 60 | - |

Data from (Weidner & Austen, 1993; Irani & Schwartz, 1994; Sperr et al., 1994; Mori et al., 1997; Buckley et al., 1998; Gotis-Graham et al., 1998; Yamada et al., 2001). Data are expressed as mean. -, Data not available; /, different values given by the authors.

**Table 3. Relative abundances of $MC_T$ and $MC_{TC}$ in normal and diseased human tissues**

| MC subtype | Synovium | | | Kidney | | | Carotid Artery | | |
|---|---|---|---|---|---|---|---|---|---|
| | Normal | Early RA | Late RA | Normal | Rejected nephrect. specimens | Rejected biopsy specimens | Normal | Early lesion | Advanced lesion |
| **$MC_T$** | 17% | 72% | 37% | 65% | 65% | 71% | 10-20% | 30-40% | 0-10% |
| **$MC_{TC}$** | 83% | 28% | 63% | 35% | 35% | 29% | 80-90% | 60-70% | 90-100% |

RA; Rheumatoid arthritis; Data from (Jeziorska et al., 1997; Gotis-Graham et al., 1998; Yamada et al., 2001)

been found at sites of active inflammation (Gotis-Graham & McNeil, 1997). This observation suggests a function for $MC_T$ in inflammatory events, whereas $MC_{TC}$ appears to be more relevant in processes of tissue remodeling (McNeil & Gotis-Graham, 2000). However, due to lack of strong

experimental evidence, the question how and why an MC develops into $MC_T$ and $MC_{TC}$ remains a matter of debate.

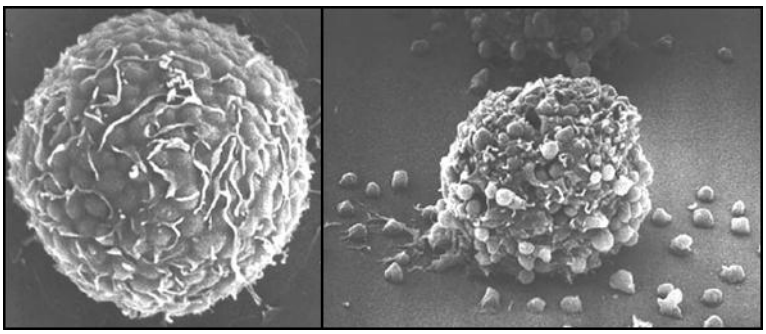

**Figure 2. Scanning electron micrographs of resting and degranulating rat serosal MCs**. Under normal conditions, MCs are found in tissues in their resting state storing the various neutral proteases in their cytoplasmic granules (*Left panel*). However, upon activation of an appropriate stimulus, such as crosslinking of receptor-bound IgE by an antigen, MCs acutely exocytose their preformed mediators in a process called degranulation (*Right panel*). Whereas histamine and other soluble mediators diffuse away, a fraction of the exocytosed neutral proteases remain bound to heparin, forming a proteolytically active granule remnant (Kokkonen & Kovanen, 1990).

### 2.4. Mast cell development

The origin of tissue MCs remained unclear for a long time; in the decades after the discovery of MCs, they were incorrectly identified as deriving from T cells, plasma cells, monocytes, basophils, fibroblasts, mesenchymal cells, and even from endothelial cells (Burnet, 1977; Zucker-Franklin, 1980; Czarnetzki et al., 1982; Metcalfe et al., 1997; Schwartz & Huff, 1998). However, to date, it is clear that MCs, like basophils and other blood cells, derive from hematopoietic stem cells in the bone marrow. MCs are rather unique among cells of hematopoietic origin in that they undergo only part of their differentiation in the bone marrow, with the bulk differentiation occurring in peripheral tissues under the influence of the local microenvironment (Okayama & Kawakami, 2006).

The bone marrow origin of MCs was first demonstrated through a series of *in vivo* reconstitution studies using genetically MC-deficient mutant mice (Kitamura et al., 1978; Kitamura et al., 1981). The $WBB6_{F1}$-$W/W^V$ mouse is MC-deficient due to a homozygous mutation at the white spotting locus (*W*); however, these mice are capable of developing MCs from homografts of bone marrow of their normal littermates. In contrast, WCB6F1-$S1/S1^d$ mice that are MC-deficient due to homozygous mutation at the steel locus (*S1*) do not develop MCs under any circumstances (Kitamura & Go, 1979). However, the $S1/S1^d$ mouse bone marrow cells develop into MCs after

intravenous injection into $W/W^v$ mice (Kitamura & Go, 1979). These and other results suggested that $W/W^v$ mice have abnormal bone marrow progenitors for MC, whereas $Sl/Sl^d$ mice have normal bone marrow progenitors, but the microenvironment that induces MC differentiation is defective. The molecular explanations for these MC-deficiencies were provided when the W and S1 genes were cloned. The W locus contains c-kit proto-oncogene, which encodes the KIT receptor tyrosine kinase expressed on MCs and their progenitors, whereas the S1 locus encodes a hematopoietic growth factor called stem cell factor (SCF), steel factor, or kit ligand (KITLG) (Chabot et al., 1988; Geissler et al., 1988; Anderson et al., 1990; Flanagan & Leder, 1990), which is highly expressed on the surface of fibroblasts and stromal cells and also expressed in a non-membrane-bound secretable form (Witte, 1990).

### *2.4.1. Bone marrow phase and mast cell progenitors*

In humans, pluripotent hematopoietic stem cells in the bone marrow first differentiate into common $CD34^+$ myeloid progenitor cells (Kirshenbaum et al., 1991). MCs can be derived from this population of $CD34^+$ progenitor cells in the presence of KITLG, so defining human MC progenitors as $CD34^+/KIT^+$ cells (Kirshenbaum et al., 1999). Since also monocytes and granulocytes can be cultured from $CD34^+/KIT^+$ progenitors, several hypotheses about the relationship between MCs and other hematopoietic cells have been raised (Burnet, 1977; Zucker-Franklin, 1980; Czarnetzki et al., 1982) However, in 1999, Kirshenbaum and colleagues noted that when the membrane-associated aminopeptidase N (CD13) was expressed on the $CD34^+/KIT^+$ cells, it served as a marker for a progenitor population that included MC and monocyte precursors, and thus distinguished this population of progenitors from granulocyte-committed precursors (Kirshenbaum et al., 1999). Moreover, lack of the monocyte-associated lipopolysaccharide receptor subunit (CD14) was earlier shown to distinguish MC progenitors from the population of progenitors that give rise to monocytes (Agis et al., 1993). In conclusion, circulating MC progenitors can be defined as $CD34^+/KIT^+/CD13^+/CD14^-$ to distinguish them from the $CD34^+/KIT^+/CD13^+/CD14^+$ and the $CD34^+/KIT^+/CD13^-$ progenitors that give rise to monocytes and granulocytes, respectively (Agis et al., 1993; Kirshenbaum et al., 1999). Observations of other groups that have characterized human MC progenitors support the conclusion that human MCs derive from bone marrow progenitors as a default lineage, not functionally related to any other lineage (Rottem et al., 1994; Kempuraj et al., 1999).

### *2.4.2. Peripheral differentiation phase*

From the circulating blood, the MC progenitors are recruited into various peripheral tissues. The processes of homing and migration of MCs to peripheral tissues have been extensively studied in mice. It has become apparent that MC homing in mice is a precisely controlled process, where integrin adhesion molecules play an important role (Hallgren & Gurish, 2011). Human MC progenitors have been reported to use α4β1-integrin for adhesive interactions with human vascular endothelium (Boyce et al., 2002). Furthermore, human MC progenitors express several chemokine receptors, CXCR2, CCR3, CXCR4, and CCR5, and respond to their ligands IL-8, eotaxin, stromal cell-derived factor (SDF)-1α, and macrophage inflammatory protein (MIP)-1α, respectively *in vitro* (Ochi et al., 1999).

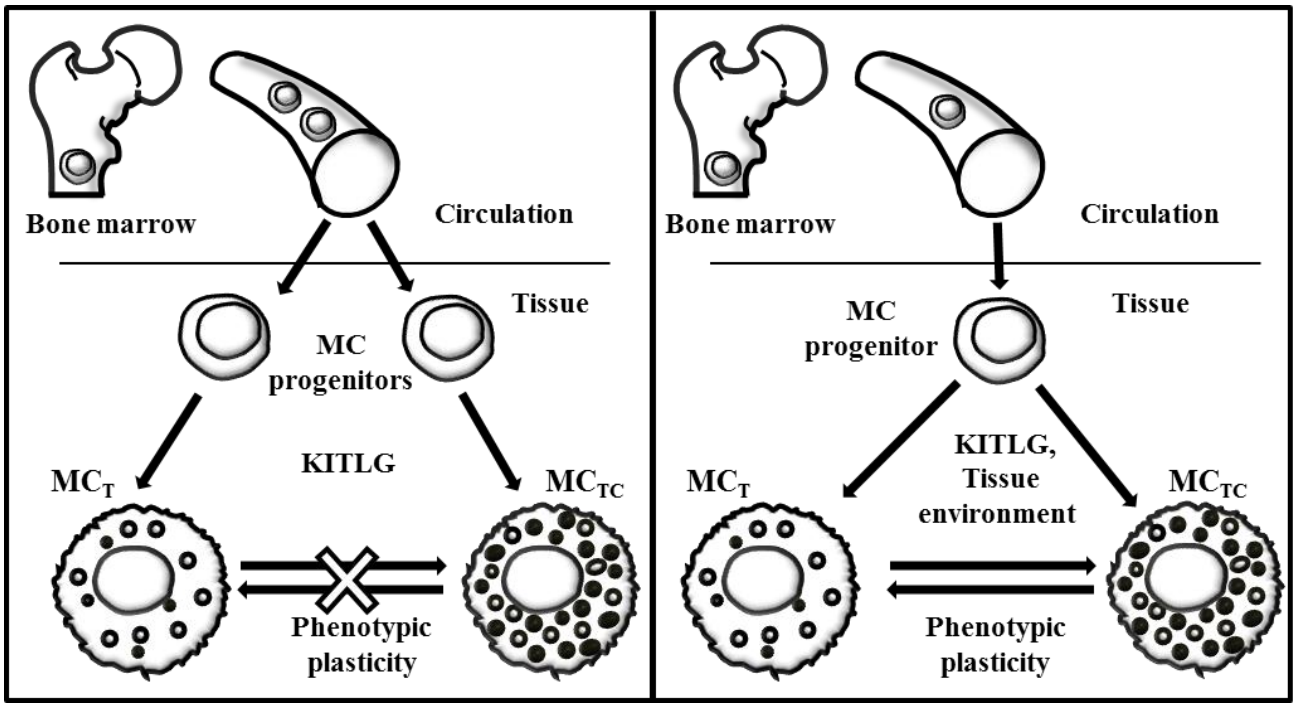


**Figure 3. Two models for human MC development.** MCs derive from pluripotent hematopoietic $CD34^+$ stem cells in the bone marrow, leave the bone marrow as committed $CD34^+/KIT^+$ progenitor cells, and migrate through the bloodstream into peripheral tissues to undergo terminal differentiation under the influence of local growth factors and cytokines, most importantly KITLG. Two models for the development of $MC_T$ and $MC_{TC}$ have been suggested. First, $MC_T$ and $MC_{TC}$ derive from two distinct progenitors with irreversibly predetermined protease phenotypes, and no transdifferentiation between the two MC subtypes exists (*Left panel*). Second, $MC_T$ and $MC_{TC}$ derive from a common progenitor cell, and microenvironmental factors, such as cytokine milieu, ultimately determine the protease phenotype. Thus, $MC_T$ and $MC_{TC}$ can switch phenotypes along with changes in the local microenvironment ("Phenotypic plasticity") (*Right panel*). Adapted from (Nigrovic & Lee, 2013).

The details of human MC differentiation in local tissue microenvironments are not well understood, and the developmental relationship between $MC_{TC}$ and $MC_T$ is particularly controversial: Are $MC_{TC}$ and $MC_T$ committed subsets deriving from two distinct progenitors with irreversibly predetermined protease phenotypes, or do they derive from a common progenitor cell and represent functional states that MCs assume under the influence of the local microenvironment (Fig. 3)?

It has become obvious however that KITLG is the most important differentiation signal to MCs from the local microenvironment. KITLG elicits its functions, such as promotion of cell–cell and cell–substratum adhesion, proliferation, differentiation, maturation, and survival of MCs (Okayama & Kawakami, 2006), through KIT, which is expressed widely on hematopoietic lineages early in differentiation, however, among mature lineages, only MCs express it at high levels (Ribatti & Crivellato, 2014). Stimulation of MCs by KITLG also blocks apoptosis, induces chemotaxis, and may activate MCs directly to release mediators. It is clear that the presence of KITLG is an absolute requirement for MC development and viability both *in vivo* and *in vitro*. Thus, mice with defects in KITLG ($Sl/Sl^d$ mutants) or KIT ($W/W^v$ mutants) are strikingly deficient in mature tissue MCs. On the other hand, clonal MCs obtained from patients with systemic mastocytosis characterized by uncontrolled MC proliferation commonly have autoactivating mutations of KIT (Carter et al., 2014).

Several factors are capable of regulating the KITLG-dependent MC development in both humans and mice. For instance, T lymphocytes have a profound impact on the phenotype and survival of local tissue MCs; intestinal biopsy specimens of human patients suffering from reduced T cell numbers due to congenital immunodeficiency or acquired immunodeficiency syndromes (AIDS) have shown that a reduction in T cell numbers is associated with strikingly reduced numbers of $MC_T$, whereas $MC_{TC}$ are present in normal numbers (Irani et al., 1987; Bentley et al., 1996). Similarly, large numbers of $MC_T$ are identified in the inflamed synovium of patients with rheumatoid arthritis, typically in the regions rich in infiltrating leukocytes, whereas in normal synovium, $MC_{TC}$ is the predominating type observed (Gotis-Graham et al., 1997; Gotis-Graham et al., 1998). In human cell culture systems, T cell-derived cytokines, such as interleukin (IL)-3, IL-4, IL-6, and IL-9, promote proliferation and maturation of MCs (Kirshenbaum et al., 1992; Kinoshita et al., 1999; Matsuzawa et al., 2003; Lappalainen et al., 2007) and may skew the MC phenotype toward either the $MC_T$ or $MC_{TC}$ (Toru et al., 1998; Kinoshita et al., 1999). Table 4 lists factors that are known to regulate MC development, proliferation, and survival.

**Table 4. Growth factors and cytokines that regulate MC development, proliferation, and survival**

| Growth Factor/ Cytokine | Function |
|---|---|
| **KITLG** | ▪ Promotes proliferation, differentiation, survival, and cell–cell and cell–substratum adhesion (Okayama & Kawakami, 2006)<br>▪ Stimulates selective growth of MCs from hematopoietic progenitors (Irani et al., 1992; Valent et al., 1992; Kinoshita et al., 1999; Maaninka et al., 2013)<br>▪ Induces expression of various MC neutral proteases (Maaninka et al., 2013)<br>▪ Induces chemotaxis (Nilsson et al., 1994)<br>▪ Activates mediator release (Columbo et al., 1992; Sperr et al., 1993) |
| **IL-3** | ▪ Promotes development of MCs synergistically with KITLG (Kirshenbaum et al., 1992)<br>▪ Promotes growth of mature MCs (Gebhardt et al., 2002)<br>▪ Does not affect the differentiation of human MCs (Shimizu et al., 2008) |
| **IL-4** | ▪ Promotes MC maturation accompanied with increased frequency of $MC_{TC}$ phenotype (Toru et al., 1998; Ahn et al., 2000)<br>▪ Upregulates FcεRI expression (Toru et al., 1996; Xia et al., 1997; Iida et al., 2001)<br>▪ Increases mediator release (Iida et al., 2001)<br>▪ Inhibits MC growth during early stage of development (Nilsson et al., 1994)<br>▪ Induces apoptosis of MC progenitors (Oskeritzian et al., 1999)<br>▪ Inhibits early KIT expression (Nilsson et al., 1994; Kulka & Metcalfe, 2005) |
| **IL-6** | ▪ Promotes MC maturation accompanied with increased frequency of $MC_{TC}$ phenotype (Kinoshita et al., 1999; Moon et al., 2003)<br>▪ Increases histamine content (Kinoshita et al., 1999)<br>▪ Reduces/inhibits apoptosis (Kambe et al., 2001) |
| **IL-9** | ▪ Increases proliferation of MC progenitors synergistically with KITLG during early stage of MC development (Matsuzawa et al., 2003; Lappalainen et al., 2007)<br>▪ Does not affect human MC differentiation (Lappalainen et al., 2007)<br>▪ In mouse BMMCs, induce the expression of 'late-expressed' proteases mMCP-1 and mMCP-2 (Eklund et al., 1993) |
| **IFN-γ** | ▪ Inhibits MC growth and differentiation (Kirshenbaum et al., 1998)<br>▪ Inhibits early progenitor cell division (Kulka & Metcalfe, 2005)<br>▪ Promotes survival of cultured human MCs (Yanagida et al., 1996) |
| **NGF** | ▪ Promotes MC development (Welker et al., 2000) |
| **TGF-β** | ▪ Inhibits MC development (Kinoshita et al., 1999; Hjertson et al., 2003; Ishida et al., 2003) |

IFN-γ: interferon-γ; NGF: nerve growth factor; TGF-β: transforming growth factor-β

## 2.5. Tools to study mast cell protease expression

Human cell culture systems are powerful tools to study protease expression in MCs. To utilize human MCs for studies three approaches are available: 1) to obtain human MC lines, HMC-1, LAD2, or LUVA (Butterfield et al., 1988; Kirshenbaum et al., 2003; Laidlaw et al., 2011); 2) to isolate MCs from human tissues, such as the lungs, intestinal mucosa, and skin (Kulka & Metcalfe, 2010; Lorentz et al., 2015); and 3) to differentiate MCs *in vitro* from their progenitor cells present in human cord blood, peripheral blood of adult subjects, or bone marrow (Andersen et al., 2008). Regarding option 1, the MC lines represent MCs of relatively immature differentiation stages that express proteases only at low levels (Guhl et al., 2010). Regarding option 2, only a limited number of MCs can be isolated from tissues, and tissue MCs represent terminally differentiated cells, which do not usually divide in culture. Instead, *in vitro* differentiation of MCs from their progenitors ultimately yields high numbers of MCs, and thus option 3 is a method of choice when characteristics closely linked to MC development, such as protease expression, are to be investigated.

Indeed, many groups have described sophisticated protocols for the *in vitro* generation of human MCs from their progenitors. However, virtually all studies that have used such protocols to study protease expression during MC differentiation have focused solely on tryptase and/or chymase expression, whereas no studies of CPA3, cathepsin G, and granzyme B expression during MC development had been reported at the time when the present experimentation was planned. The narrow focus has its roots in the long-held concept of the existence of two and only two human MC subtypes ($MC_T$ and $MC_{TC}$), which are distinguished based on the absence or presence of chymase, respectively. However, studies that describe the presence within human tissues of MCs containing only chymase ($MC_C$) (Weidner & Austen, 1993) and MCs containing tryptase and CPA3 (Abonia et al., 2010; Dougherty et al., 2010) provide strong evidence that human MC heterogeneity is more complex than initially suggested. To better understand the complexity of the phenotypic heterogeneity of human MCs, and to what extent such heterogeneity is intrinsically variable, it was important to challenge the tryptase/chymase dichotomy and to begin to appreciate the whole variety of human MC neutral proteases.

# 3. AIM OF THE RESEARCH

The objective of this Master's Thesis was to investigate expression pattern of various human MC neutral proteases, namely tryptase, chymase, CPA3, cathepsin G, and granzyme B during human MC development from their progenitors into mature MCs. Of particular interest was to investigate, whether human MC progenitor cells present in circulating blood differentiate in culture into different MC subtypes exhibiting heterogeneous protease phenotypes, or whether the expression of the various proteases is an intrinsic feature of all human MCs.

# 4. MATERIALS AND METHODS

## 4.1. Purification of $CD34^+$ progenitor cells from peripheral blood

For *in vitro* experiments, MC progenitors were isolated from fresh buffy coats (concentrated leukocyte suspension) prepared from peripheral blood of voluntary donors, and supplied by the Finnish Red Cross Blood Transfusion Service (Helsinki, Finland) with the acceptance of the Ethics Committee of the Finnish Red Cross. A total of 40 ml of buffy coat suspension was first diluted two-fold in $Ca^{2+}$- and $Mg^{2+}$ -free Dulbecco's phosphate-buffered saline (PBS) (Biowhittaker, Lonza, Basel, Switzerland), and then separated by density gradient centrifugation over Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden) into distinct phases that contained either erythrocytes, granulocytes, mononuclear cells, or plasma. For this purpose, 35 ml of the diluted blood sample was gently layered over 15 ml of Ficoll-Paque PLUS followed by centrifugation at 800 × g and 4°C for 30 min (without braking). The interface layer of the peripheral blood mononuclear cells (PBMCs) was then harvested and washed three times with PBS by centrifuging the cell suspension first at 800 x g and 4°C for 5 min, and then twice at 250 × g and room temperature for 5 min. Finally, the PBMCs were suspended in 50 ml of pre-chilled MACS buffer, which consisted of PBS supplemented with 0.5% bovine serum albumin (BSA) (Sigma-Aldrich, St Louis, MO, USA) and 2 mM ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich), and the total number of PBMCs was determined using Coulter Counter T-540 (Beckman Coulter, Fullerton, CA, USA). After determination of the total cell yield, the PBMCs were sedimented by centrifuging them at 300 × g and room temperature for 10 min, and were subsequently used for $CD34^+$ cell separation.

$CD34^+$ progenitor cells were enriched using positive immunomagnetic selection, a method that includes $CD34^+$ cell separation kit and a magnetic $LS^+$ separation column, according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA, USA). For this purpose, PBMCs were resuspended in pre-chilled MACS buffer at a concentration of 300 µl per $1 \times 10^8$ cells. The Fc-receptors were then blocked with human IgG followed by labeling of the cell surface antigens with CD34 hapten antibody (100 µl per $1 \times 10^8$ cells) at 4 ºC for 15 min. After incubation, the cells were washed with pre-chilled MACS buffer by centrifuging them at 300 × g and room temperature for 6 min, resuspended in MACS buffer at a concentration of 400 µl per $1 \times 10^8$ cells, and incubated with CD34 anti-hapten MicroBeads (100 µl per $1 \times 10^8$ cells) at 4ºC for 15 min. After washing with MACS buffer at 300 × g and room temperature for 6 min, the cells were resuspended in MACS buffer at a concentration of 500 µl per $1 \times 10^8$ cells, and finally passed through the MACS

separation column placed in the magnetic field of the MACS separator. The magnetically labeled $CD34^+$ cells were retained in the column, while the other cell types were eluted and discarded by washing the column four times with 4 ml of pre-chilled MACS buffer. After the washes, the column was removed from the magnetic field, and the $CD34^+$ cells were eluted as the positively selected cell fraction with 5 ml of pre-chilled MACS buffer and washed once by centrifuging them at 300 × g and room temperature for 6 min. Finally, the $CD34^+$ cells were resuspended in 2 ml of culture medium (described below), stained with 0.4% Trypan blue, and counted for evaluation of the cell yield.

### 4.2. Cell culture

The isolated $CD34^+$ progenitor cells were grown on 12-well culture plates (Falcon, BD Biosciences, San José, CA, USA) under serum-free conditions according to a recently published protocol (Lappalainen et al., 2007). The basal culture medium consisted of Iscove's Modified Dulbecco's Medium (IMDM) with L-Glutamine and 25 mM Hepes (Biowhittaker, Lonza) supplemented with 100 U/ml penicillin (Biowhittaker, Lonza), 100 μg/ml streptomycin (Biowhittaker, Lonza), 100 μM β-mercaptoethanol (Sigma-Aldrich), 100 ng/ml recombinant human (rh)KITLG (PeproTech, Rocky Hill, NJ, USA), and 20% serum substitute, BIT 9500 supplement (containing BSA, human recombinant insulin and human transferrin) (Stem Cell Technologies, Vancouver, British Columbia, Canada). The basal culture medium was supplemented sequentially with rhIL-3 (5 ng/ml), rhIL-9 (15 ng/ml), rhIL-6 (50 ng/ml) (PeproTech, Rocky Hill, NJ, USA) as well as human low-density lipoprotein (LDL) (10 μg/protein/ml) that was prepared from the plasma of healthy volunteers supplied by Finnish Red Cross as described previously (Lappalainen et al., 2011). Medium changes were performed twice a week during the first three weeks of culture, and weekly thereafter. For that purpose, culture medium was first spun at 300 × g and room temperature for 6 min to sediment the cells, was then gently aspirated and replaced by fresh medium, after which the cells were transferred to grow on new culturing plates. During the culture period the concentration of the cells were kept around $0.5 \times 10^6$ cells/ml. For the first four weeks, the cells were cultured at 37°C in a humidified incubator flushed with a mixture of 5% $O_2$, 5% $CO_2$, and 90% $N_2$ and thereafter under normoxic conditions (21% $O_2$ and 5% $CO_2$) at 37°C. Cell viability was determined weekly by Trypan blue exclusion test. The cells were cultured for a total of 9 weeks, after which all cells were shown to express mature MC phenotype (Lappalainen et al., 2007).

### 4.3. Flow cytometry

Expression of tryptase, chymase, cathepsin G, granzyme B, and CPA3, as well as histidine decarboxylase (HDC) as an indicator for cellular histamine, was followed on weekly basis during the MC development by flow cytometry using a fluorescence-activated cell sorter (FACS) LSR II (BD Biosciences). Double staining with KIT (or c-KIT) was used to distinguish MCs from other cell types, for example from macrophage/monocytes and basophils. The antibodies and their concentrations are listed on Table 5.

For flow cytometry, 2 x $10^5$ cells per sample were collected and sedimented by centrifuging them at 300 x g and room temperature for 6 min. The cells were then resuspended in pre-chilled FACS buffer (sterile-filtered through 0.2 μm pore size membrane, Corning, NY, USA) consisting of 0.5% BSA (Sigma-Aldrich) and 0.025% sodium azide (Sigma-Aldrich) in PBS, and then centrifuged at 300 × g and 4ºC for 6 min. Next, the cells were resuspended in FACS buffer at a concentration of 2 × $10^5$ cells/50 µl and transferred to a 96-well Sarstedt plate (Sarstedt Microtest 96-well Plates with V-shape bottom). The cells were fixed by adding 50 µl of 4% paraformaldehyde (PFA, Sigma-Aldrich) in PBS and by incubating for 15 min at room temperature, after which the cells were washed once with 150 µl of FACS buffer at 300 × g and room temperature for 5 min, and resuspended in a 50 µl of FACS buffer. For double-staining of the cell surface antigens FcεRI and KIT, the cells were first incubated with phycoerythrin (PE)-conjugated mouse anti-human FcεRI, or its negative control for 30 min at 4°C, after which the excess antibody was washed by centrifuging the cells with 250 µl of FACS buffer at 300 × g and room temperature for 5 min. The cells were then resuspended in 50 µl of FACS buffer and incubated with allophycocyanin (APC)-conjugated mouse anti-human CD117 (KIT), or its negative control for 30 min at 4°C. The excess antibody was again washed by centrifuging the cells with 250 µl of FACS buffer at 300 × g and room temperature for 5 min, and finally, the cells were resuspended in 250 µl of FACS buffer.

For double-staining of the proteases/HDC and KIT, the fixed cells were permeabilized in 100 µl of 0.5% Saponin (Sigma-Aldrich) in FACS buffer by incubating them for 20 min at room temperature. Next, primary antibodies against tryptase, chymase, cathepsin G, CPA3, granzyme B, and HDC, or their negative controls, were added and incubated with the cells for 60 min at 4°C. Excess antibodies were washed by centrifuging the cells twice with 200 µl of 0.25% saponin in FACS buffer at 300 x g and room temperature for 5 min. Next, the cells were resuspended in 50 µl of

0.25% saponin in FACS buffer and secondary antibodies were added and incubated with the cells for 40 min at 4°C. Excess antibodies were washed by centrifuging the cells twice with 200 µl of 0.25% Saponin in FACS buffer at 300 x g and room temperature for 5 min. The cells were resuspended in 80 µl of FACS buffer and stained against KIT by incubating them with APC-conjugated mouse anti-human CD117, or its negative control for 30 min at 4°C. Finally, the cells were washed by centrifuging them twice with 200 µl of FACS buffer at 300 × g and room temperature for 5 min. Just before analysis, the cells were transferred to 5 ml FACS tubes (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA) and 250 µl of pre-chilled FACS buffer was added to each sample. The FACS analysis was performed with $1.0 \times 10^4$ cells per sample by FlowJo software from TreeStar Inc. (Ashland, OR, USA). To eliminate any contaminating cells from being analyzed, $KIT^+$ cells were first gated followed by examination of FcεRI or intracellular proteases/HDC (Fig. 4).

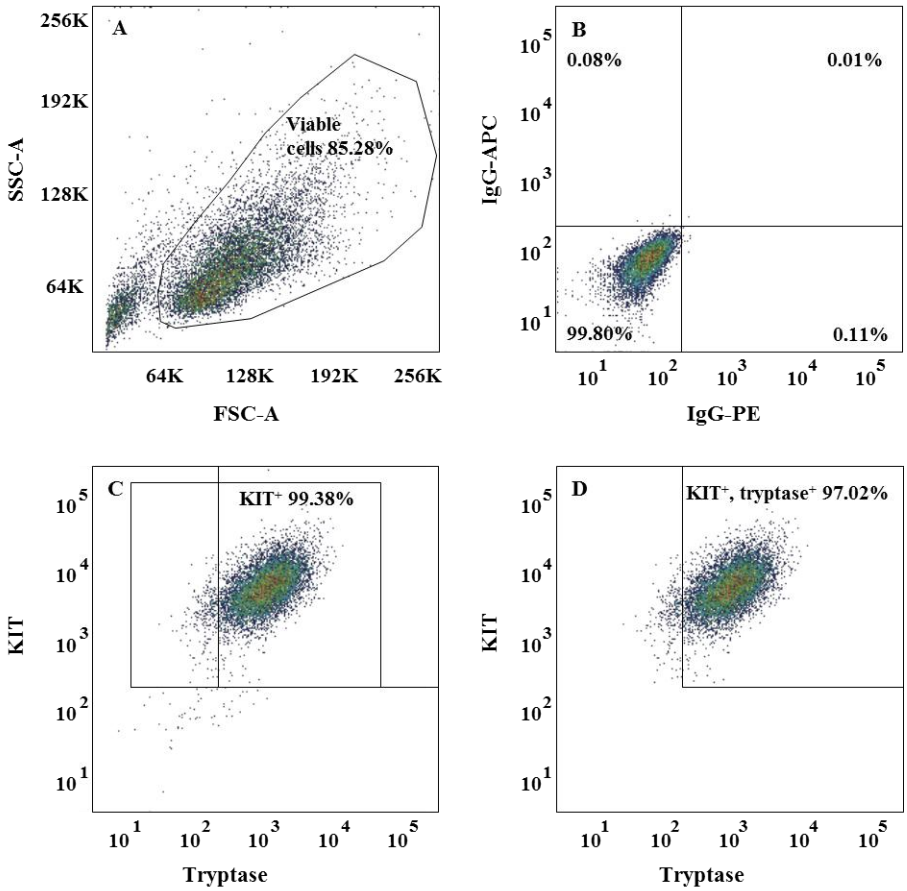


**Figure 4. A representative plot of flow cytometric analysis of the human peripheral blood $CD34^+$ progenitor-derived mast cells at 6 weeks of culture.** Quadrant plots were created to evaluate the proportion of cells double-positive for KIT and the investigated proteases/HDC/FcεRI. Viable cells were determined according to straight and forward scatters (SSC and FSC) (A). Fluorescence thresholds for positive staining were set by irrelevant isotype-matched immunoglobulins (B). Cells were then gated by their high surface expression of KIT (C), which excluded any contaminating cell types from being analyzed. Finally $KIT^+$ cells were analyzed for FcεRI or intracellular proteases/HDC (D).

## Table 5. Antibodies used for FACS and immunocytochemical stainings of mast cell proteases

| Antibody | Isotype | Clone | Manufacturer | Concentration | Final concentration | Label |
|---|---|---|---|---|---|---|
| **Primary antibodies** | | | | | | |
| **CD117/KIT** | | | | | | |
| Mouse anti-hu CD117 mAb | IgG1 | YB5.B8 | eBiosciences | 0.025 mg/ml | 5 µg/ml | APC |
| Mouse anti-hu CD117 mAb | IgG1 | YB5.B8 | BD Pharmingen | 0.2 mg/ml | 4 µg/ml | APC |
| **Isotype-matched negative control** | | | | | | |
| Mouse IgG isotype control | IgG1 | MOPC-21 | BD Pharmingen | Not available | 1:5 | APC |
| **IgERI** | | | | | | |
| Mouse anti-hu IgERI mAb | IgG1 | CRA-1 | BioSite | Not available | 1:10 | PE |
| Mouse anti-hu IgERI mAb | IgG1 | AER-37 | eBiosciences | Not available | 1:10 | PE |
| **Isotype-matched negative control** | | | | | | |
| Mouse IgG negative control | IgG1 | 203 | ImmunoTools | Not available | 1:10 | PE |
| Mouse IgG negative control | IgG1 | Not applicable | AbD Serotec | Not available | 1:10 | PE |
| **Granzyme B** | | | | | | |
| Mouse anti-hu Granzyme B mAb | IgG1 | GB11 | ImmunoTools | Not available | 1:10 | PE |
| Mouse anti-hu Granzyme B mAb | IgG1 | GB11 | AbD Serotec | Not available | 1:10 | PE |
| Rabbit anti-hu Granzyme B pAb* | Not applicable | Not applicable | Abcam | Not available | 1:10 | |
| **Isotype-matched negative control** | | | | | | |
| Mouse IgG negative control | IgG1 | 203 | ImmunoTools | Not available | 1:10 | |
| Mouse IgG negative control | IgG1 | Not applicable | AbD Serotec | Not available | 1:10 | PE |
| **Tryptase** | | | | | | |
| Mouse anti-hu tryptase mAb | IgG1 | AA1 | AbD Serotec | 1 mg/ml | 10 µg/ml | |
| **Chymase** | | | | | | |
| Mouse anti-hu chymase mAb | IgG1 | B7 | Chemicon/Millipore | 1.6 mg/ml | 3.2 µg/ml | |
| **Isotype-matched negative control** | | | | | | |
| Mouse IgG negative control | IgG1 | Not applicable | AbD Serotec | Not available | 1:30 | |
| **Cathepsin G** | | | | | | |
| Rabbit anti-hu cathepsin G pAb | Not applicable | Not applicable | Abcam | Not available | 1:100 | |
| **Isotype-matched negative control** | | | | | | |
| Rabbit IgG negative control | Not applicable | Not applicable | Jackson laboratories | 1 mg/ml | 0.4 µg/ml /1 µg/ml | |
| **CPA3** | | | | | | |
| Rabbit anti-hu CPA3 pAb | Not applicable | Not applicable | Sigma-Aldrich | 0.11 mg/ml | 0.22 µg/ml | |
| Rabbit anti-hu CPA3 pAb | Not applicable | Not applicable | Sigma-Aldrich | 0.01 mg/ml | 0.4 µg/ml (2 µg/ml*) | |
| **HDC** | | | | | | |
| Rabbit pAb to HDC | Not applicable | Not applicable | Abcam | 0.2 mg/ml | 2 µg/ml | |
| **IgG isotype control** | | | | | | |
| Rabbit IgG negative control | Not applicable | Not applicable | Jackson laboratories | 11 mg/ml | 2.2 µg/ml (10 µg/ml*) | |
| Rabbit IgG negative control | Not applicable | Not applicable | Jackson laboratories | 1 mg/ml | 0.4 µg/ml/ 1 µg/ml | |
| **Secondary antibodies** | | | | | | |
| Donkey pAb to Rabbit IgG H&L | $F(ab)_2$ fragment | Not applicable | Abcam | 0.5 mg/ml | 5 µg/ml | PE |
| Sheep anti-rabbit | Full IgG | Not applicable | AbD Serotec | NA | 1:5 | PE |
| Goat anti-mouse IgG | Full IgG | Not applicable | BD Pharmingen | 0.2 mg/ml | 2 µg/ml | PE |
| Goat anti-mouse IgG | $F(ab)_2$ fragment | Not applicable | Molecular Probes | 2 mg/ml | 4 µg/ml | FITC |
| Goat anti-rabbit IgG | Full IgG | Not applicable | Molecular Probes | 2 mg/ml | 4 µg/ml | FITC |

* Used only for immunofluorescence microscopy

### 4.4. Quantitative real-time reverse-transcription PCR

To evaluate the mRNA expression levels of tryptase, chymase, CPA3, cathepsin G, granzyme B as well as HDC, qRT-PCR was used. The protease and HDC expressions in developing MCs were followed on a weekly basis at compatible time points to flow cytometric analysis.

#### *4.4.1. Total RNA isolation*

Total RNA was isolated from developing MCs on a weekly basis, starting at week 1 of culture, with RNeasy mini kit according to the manufacturer's instructions (Qiagen Inc., Valencia, CA, USA). The developing MCs ($0.5 \times 10^6$ cells per culture) were harvested and lysed in 350 µl of RLT PLUS Buffer, supplemented with 1% β-mercaptoethanol (Sigma-Aldrich). The formed cell lysate was homogenized by flicking the cell suspension back and forth 5 times through a 19-gauge needle. To precipitate the RNA present in the cell lysates, 350 µl of 70% ethanol was added to the lysates and mixed carefully. Next, the solution was transferred into a RNA spin column in a 2 ml collection tube and centrifuged at 10 000 × rpm for 15 s [all centrifugation steps were performed at room temperature with Centrifuge 5424 (Eppendorf AG, Hamburg, Germany)]. The spin column was washed by centrifuging with 350 µl of the RW1 buffer at 10 000 × rpm for 15 s. The flow-through was discarded, and 80 µl of 1:8 RNase-free DNase I in RDD buffer was added to RNA spin column membrane to eliminate any remnants of genomic DNA. After 15-min incubation at room temperature, 350 µl of the RW1 buffer was added to the RNA spin column and the column was centrifuged at 10 000 × rpm for 15 s. The flow-through volume was discarded, and 500 µl of RPE buffer was added to the spin column twice, so that the column was first centrifuged at 10 000 x rpm for 15 s and then at 10 000 × rpm for 2 min. The flow-through volume was again discarded, and the spin column was transferred to another 2 ml collection tube and centrifuged at 10 000 × rpm for 1 min to dry the spin column membrane. Finally, RNA was eluted from the column with 30 µl of RNase-free water by centrifuging the column at 10 000 × rpm for 2 min in 1.5 ml collection tube. The RNA concentration was then measured with NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Thermo Scientific, Wilmington, DE, USA) in a sample volume of 1.5 µl.

### *4.4.2. Complementary DNA synthesis from total RNA*

For complementary DNA (cDNA) synthesis, 250 ng of total RNA per sample was reverse transcribed in a reaction mixture consisting of 0.01 μM dNTP mix (Promega Madison, WI, USA), 200 ng random primers (Promega), 5X First-Stand Buffer (Promega), 20 U RNase Inhibitor (Promega), 100 U M-MLV RT (Promega), and RNase-free water in a final volume of 20 μl. First the RNA, dNTP mix, random primers, and RNase-free water was added to a 0.2 ml PCR tubes, incubated at 65˚C for 5 min, and immediately placed on ice. Next, 5X First-Strand Buffer, RNase Inhibitor, M-MLV RT enzyme, and RNase-free water were added to each sample tube and mixed gently, after which the samples were reverse transcribed as follows: primer annealing at 25˚C for 8 min, followed by first strand synthesis at 48˚C for 55 min, and termination at 85˚C for 10 min. Finally, the samples were chilled at 4˚C, spun down by brief centrifugation at room temperature, diluted four-fold in PCR grade water, and used for qRT-PCR.

### *4.4.3. qRT-PCR*

A TaqMan, or alternatively SYBR Green, analysis was performed using ABI PRISM 7500 sequence detector (Applied Biosystems, Foster City, CA). Five microliters of the diluted cDNA from 250 ng of total RNA was amplified in duplicates using TaqMan Universal PCR Master Mix or Power SYBR Green Master Mix (both from Applied Biosystems) together with 400 nM primers and 200 nM FAM-labeled fluorogenic probes (SYBR Green assays were performed without a fluorogenic probe) in a final volume of 25 μl. The forward and reverse oligonucleotide primers as well as TaqMan probes (Table 6) were in-house designed and synthesized by Biomers (Ulm, Germany). The thermocycling conditions for the qRT-PCR assay consisted of an initial step of

**Table 6. Primers and probes used in quantitative real time-PCR**

| Gene | SS Primer | AS Primer | Probe |
|---|---|---|---|
| Tryptase | CGATGTGGACAATGATGAGC | CGCCAAGGTGGTATTTTGC | |
| Chymase | TGCTCATTGTGCAGGAAGGTC | ACCTCAAGCTTCTGCCATGTG | |
| CPA3 | CCAGATGCTATTGTTTCCCTATGG | GTAGCGGGTTTCATATCGAGTTG | FAM -CCATGAGGACTTGGCCAAAGTTGCA -BHQ-1 |
| Cathepsin G | GGTTCCTGGTGCGAGAAGAC | GATGTGTTGCTGGGTGTTTTC | FAM–AAGCAATATAAATGTCACCCTGGGCGC-BHQ-1 |
| Granzyme B | GGAAGATCGAAAGTGCGAATC | CTGGGCCACCTTGTTACACA | FAM-CCTCCAGAGTCCCCCTTAAAGGAAGTCT-BHQ-1 |
| HDC | CTGATGCCATCCAACTGCTTG | TTGCCAACCAGTCCATGAG | FAM-TCACCTGGGCATCCAGCCCTG-BHQ-1 |
| 18s | CGGCTACCACATCCAAGGAA | GCTGGAATTACCGCGGCT | FAM-TGCTGGCACCAGACTTGCCCTC-BHQ-1 |

95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, and annealing and extension at 60°C for 1 min. PCR-grade water was included in each assay to replace the cDNA template as a no template control. For data normalization an endogenous control, 18S ribosomal (r)RNA was determined for controlling cDNA input and PCR amplification.

The data was developed with Sequence Detector System software (version 1.4, Applied Biosystems), the threshold value (Ct) of a sample was selected according to the manufacturer's guidelines, and the relative units were calculated by a comparative *Ct* method. First, the difference between the *Ct* of the 18S rRNA and the corresponding target gene was calculated for $^{\Delta}Ct$. For the calibrator $^{\Delta}Ct$, the average *Ct* of 18S rRNA from each sample was subtracted from the calibrator *Ct* value of 40, which was obtained from the no template control (molecular-grade water). Next, the calibrator $^{\Delta}Ct$ was subtracted from the $^{\Delta}Ct$ of each experimental sample to obtain the $^{\Delta\Delta}Ct$. Finally, the amount of target normalized to an endogenous control was calculated by the equation $2^{-\Delta\Delta Ct}$ (Livak & Schmittgen, 2001).

### 4.5. Immunocytochemical stainings of mast cell proteases

Identification of tryptase, chymase, CPA3, cathepsin G, and granzyme B in mature MCs was performed by immunocytochemical stainings. The antibodies used for immunocytochemical stainings are listed on Table 5. Ten thousand MCs per sample were centrifuged onto glass slides by Cytospin (Shandon, Thermo Scientific) at 600 x rpm and room temperature for 6 min. After allowing the slides to air-dry, the cells were fixed with methanol supplemented with 10% MeS Buffer that consisted of 100 mM MeS (2-morpholinoethanesufonic acid), 1 mM EGTA (ethylene glycol tetraacetic acid), and 1 mM $MgCl_2$, at room temperature for 5 min. Next, the samples were washed three times with PBS for 5 min each, after which the non-specific binding sites of MCs were blocked with 80 µl of 3% goat serum in PBS for 30 min at room temperature. Next, the serum was replaced by 80 µl of the primary antibodies against tryptase, chymase, CPA3, cathepsin G, and granzyme B, or by 80 µl of their isotype-matched negative controls, and the slides were incubated for 120 min in a humidified chamber at 4ºC. Excess antibody was washed with 0.05% Tween 20 (Sigma-Aldrich) in PBS for 5 min, and then two times 5 min with PBS only. Next, the secondary antibodies were added to the slides and incubated for 60 min in the humidified chamber at room temperature. Excess antibody was again washed with 0.05% Tween 20 in PBS and then two times 5 min with PBS only, and the cell nuclei were quickly stained with 20 µg/ml of DAPI (4',6-

diamidino-2-phenylindole, Sigma-Aldrich). Finally, the samples were washed once with distilled water, mounted with Fluorescent mounting media (Sigma-Aldrich) and cover slips, and inspected under Nikon Eclipse E600 fluorescence microscopy (Nikon Instruments Europe B.V., Amsterdam, Netherlands). The pictures were taken with Spot RT and by SpotAdvenced software both from Diagnostics Instruments (Sterling Heights, MI, USA).

### 4.6. Cellular histamine

The expression of cellular histamine was measured on a weekly basis using enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (IBL, Boise, ID, USA). The cells, $0.15 \times 10^6$ MCs per sample, were harvested, sedimented with their culture medium (at 300 × g and room temperature for 5 min), and lysed in 50 µl of 0.5% Triton-X 100 (Sigma-Aldrich) in water. The cell lysates were then diluted in water, and 50 µl of each diluted sample and of each standard were transferred into 5 ml glass tubes. Next, 50 µl of Indicator Buffer and 10 µl of Acylation Reagent were added into each tube, and the samples were immediately mixed and incubated at room temperature for 30 min. After incubation, 2 ml of Assay Buffer (diluted five-fold in water) was added into each tube, which were then mixed thoroughly. Next, 50 µl of each acylated standard and acylated sample were transferred into the respective wells of a microtiter plate, after which 50 µl of freshly prepared Enzyme Conjugate and 50 µl of Histamine Antiserum were added into each well. The samples were then incubated for 180 min on an orbital shaker at room temperature, and after incubation, washed 4 times with 250 µl of freshly prepared Wash Buffer. The excess solution was removed and 100 µl of 3,3′,5,5′-Tetramethylbenzidine (TMB) Substrate Solution was added to the samples and standards, and incubated on an orbital shaker at room temperature for 20 min. The reaction was stopped by adding 100 µl of TMB Stop Solution into each well, and the optical densities at 450 nm were measured immediately with a Wallac Victor 3 (Perkin-Elmer, Turku, Finland).

# 5. RESULTS

## 5.1. Cell culture

$CD34^+$ progenitor cells were derived from the peripheral blood of healthy volunteers and cultured under a recently developed protocol in the presence of rhKITLG and various cytokines to allow them to differentiate into mature $MC_{TC}$ type of MCs during 9 weeks of culture (Lappalainen et al., 2007). The yield of PBMC from one buffy coat was, on average, $490 \times 10^6$ cells (n=7 donors), of which, 0.3% on average were ultimately cells recovered as $CD34^+$ after the positive immunomagnetic selection. The culture was initiated with approximately $1.5 \times 10^6$ $CD34^+$ cells. During the culture, variation in the dividing potential between MCs of different donors was observed, and accordingly, the time an individual donor contributed to the analyses ranged from 5 to 9 weeks.

## 5.2. Antigen expression by flow cytometry

### *5.2.1. KIT and FcεRI*

Expressions of KIT and FcεRI were analyzed on a weekly basis during MC development to confirm the purity of the cultures. As shown in Fig 5 (*left panel*), a small number (<10%) of primitive $CD34^+$ progenitors expressed KIT already at their isolation from the blood (at day 0). At week 1 of culture, the vast majority of the cells in culture stained positively for KIT, the proportion of $KIT^+$ cells ranging from 60% to 96% of all cells depending on the donor. By 2 weeks the proportion of $KIT^+$ cells had a tendency to drop to a range of 45% to 93%. Nevertheless, two of the donors increasingly expressed KIT at this point. After 2 weeks, the KIT-positivity again started to increase, and by 3 weeks the proportion of the $KIT^+$ cells started to equalize between different donors. At this point, 70% to 93% of the cultured cells stained positively for KIT. Beyond this time, the expression of KIT increased until up to 6 weeks, at which point more than 95% of the cells were positive for KIT among all donors. Besides the increase in proportion of $KIT^+$ cells, the positivity in terms of mean fluorescence intensity (MFI) had a tendency to increase along with culture (data not shown). The analysis of FcεRI expression was initiated at 1 week of culture, and as shown (Fig 5; *right panel*), a fraction of the primitive MC progenitors was positive for this receptor already at that time.

Expression of FcεRI increased steadily up to 3 weeks, by which time more than 90% of the MCs were characterized as FcεRI$^+$. At week 6, virtually all MCs were positive for FcεRI.

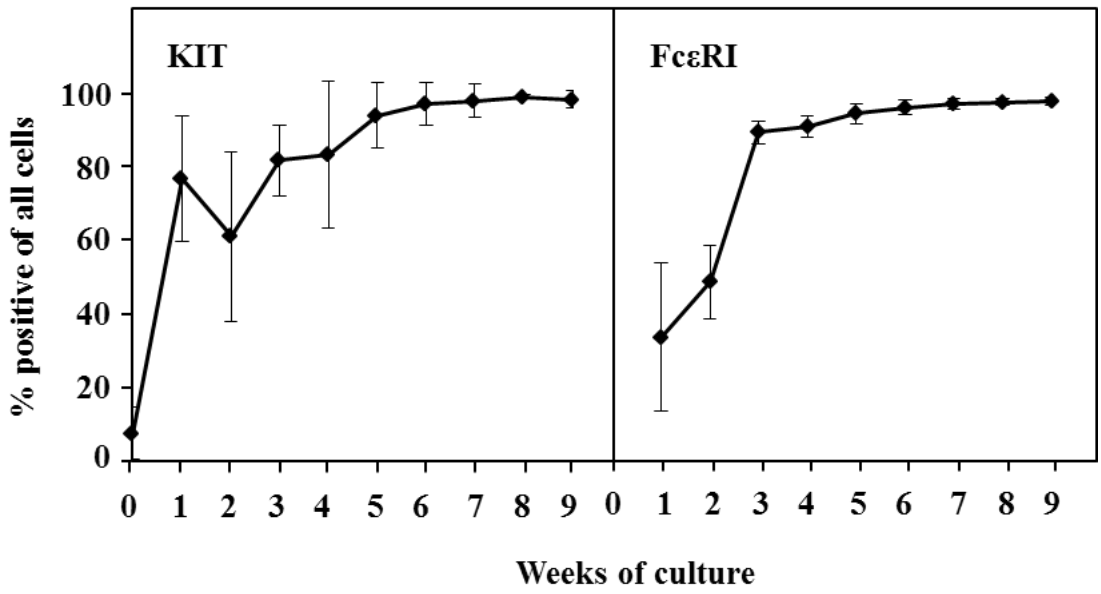


**Figure 5. Expressions of KIT and FcεRI on human peripheral blood CD34$^+$ progenitor-derived MCs during culture.** The figure illustrates the percentage of KIT- (*Left panel*) and FcεRI- (*Right panel*) positive cells of all cells in the culture at various culture weeks. The data are means ± SD from at least four donors. SD; standard deviation.

### *5.2.2. Mast cell proteases and histidine decarboxylase*

All proteases investigated herein were detected at the protein level by flow cytometry during the MC development. The flow cytometric analysis showed that each protease was expressed at detectable level in developing MC precursors as early as week 1 of culture. The amount of protease-positive cells increased relatively steadily by 3 weeks, at which point virtually all MCs were positive for tryptase and chymase (Fig 6; *left panel*). A slight delay was observed in the expressions of cathepsin G, CPA3, and granzyme B, compared to tryptase and chymase, however, by week 6-7, virtually all MCs stained positively for all the investigated proteases. Furthermore, besides the increase in the proportion of the protease-positive cells, an increase in the protease-positivity (in terms of MFI) was also observed along with culture (data not shown). No distinct protease-positive and protease-negative populations were observed at any time point during the analysis, only a single continuous population of MCs expressing each investigated protease at variable levels. The expression of HDC followed a nearly identical pattern to those of CPA3, cathepsin G, and granzyme B (Fig. 6; *left panel*).

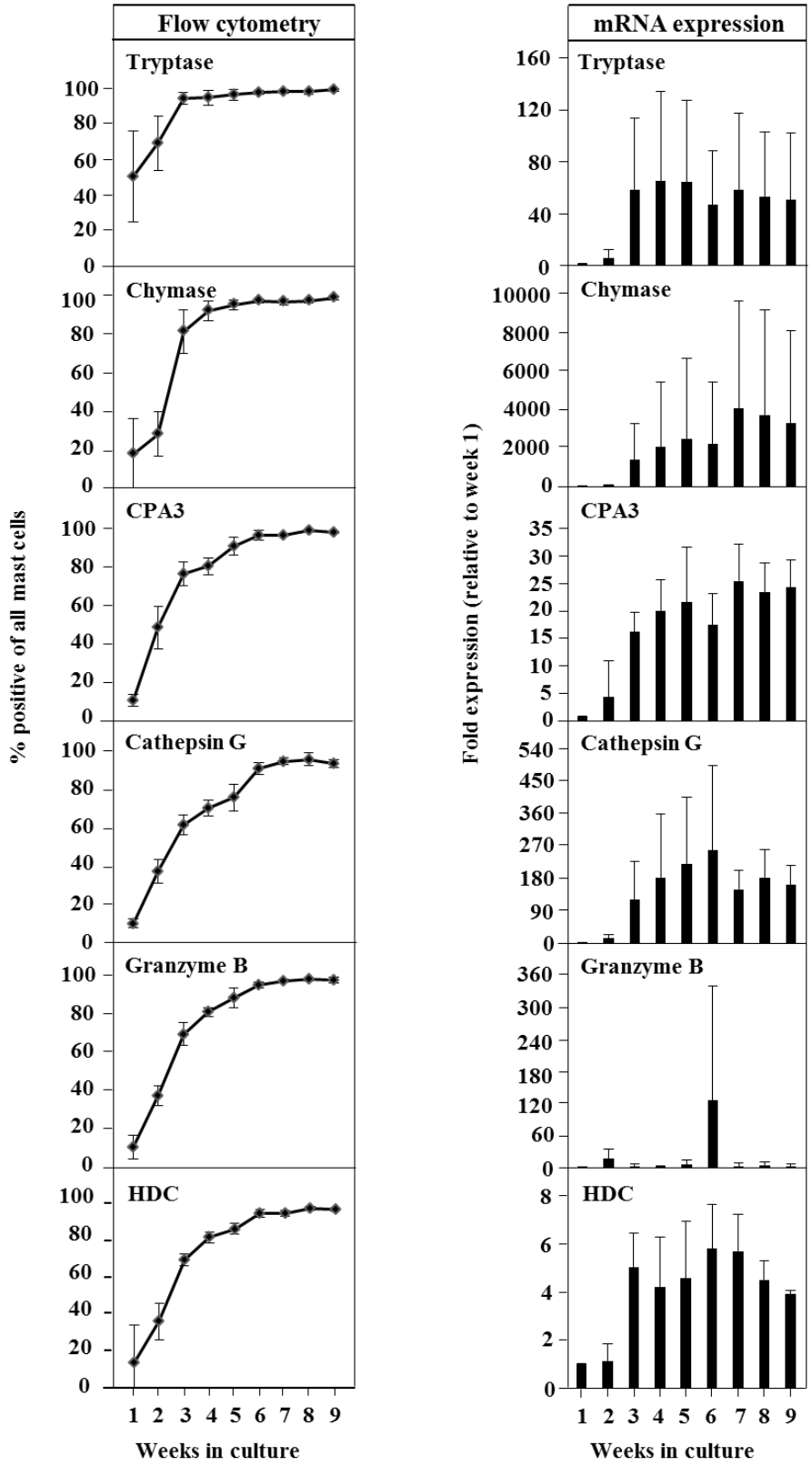


**Figure 6. Protease and histidine decarboxylase expressions in human peripheral blood CD34$^+$ progenitor-derived mast cells by flow cytometry and qRT-PCR.** The left panel illustrates the percentage of protease/HDC positive MCs of all MCs in the culture at various weeks. The right panel illustrates mRNA expression levels of the proteases/HDC at various weeks as fold increases compared to week 1. The data are means ± SD from at least four donors. SD; standard deviation.

## 5.3. mRNA analysis by qRT-PCR

### *5.3.1. Tryptase*

Tryptase mRNA levels were detected already at 1 week of culture and the levels slightly increased during the second week of culture and subsequently appeared to significantly increase at 3 weeks of culture, by which time a relatively constant level of expression was reached. In terms of absolute values (data not shown), tryptase was expressed at the highest levels, and as shown in Fig 6 (*right panel*), reached a relatively steady state level of expression at an early stage of MC development, that is, at 3 weeks of culture.

### *5.3.2. Chymase*

Chymase was also expressed at detectable level already at 1 week of culture, and the expression levels continued to increase during the second week. By 3 week, a similar tendency of remarkable increase in the mRNA expression levels that was noted with tryptase was also observed with chymase, however, the increase was significantly greater compared to that of tryptase (Fig 6; *right panel*). After 3 weeks, a relatively steady level of expression was observed without a decline in expression by 9 weeks. Of all the investigated proteases, expression levels of chymase varied the most between MCs of different donors.

### *5.3.3. Carboxypeptidase A3*

The third main MC protease, CPA3, followed similar expression pattern as observed with tryptase and chymase as being detectable at 1 week, increasing on the third week, and reaching a steady level after 3 weeks of culture (Fig 6; *right panel*). In terms of absolute values, CPA3 was expressed at relatively similar levels among the different donors. Furthermore, CPA3 was expressed at levels comparable to chymase, with the exception of the first week, during which CPA3 expression dominated over chymase.

### *5.3.4. Cathepsin G*

The mRNA for cathepsin G was expressed at detectable level at 1 week of culture and increased remarkably by 3 weeks of culture (Fig 6; *right panel*). However, the mRNA expression of cathepsin G still increased after 3 weeks, peaking at week 6 of culture, and a relatively steady level was observed at a slightly more advanced stage of cell maturation when compared to tryptase, chymase, and CPA3. In terms of absolute values, MCs expressed cathepsin G at a slightly lower level than chymase and CPA3.

### *5.3.5 Granzyme B*

The expresion of granzyme B appeared to differ from the relatively uniform expression patterns of the neutral proteases described above (Fig 6; *right panel*). However, mRNAs for granzyme B were also detected already at first week of the culture and increased during the second week of culture. However, no increase in the levels of granzyme B mRNA was observed on the third week as was noted with tryptase, chymase, CPA3, and cathepsin G. Instead, the mRNA expression had a tendency to decrease during the third week of culture. A remarkable peak at the mRNA levels was observed at 6 weeks of culture. To exclude any technical problems in the assay, granzyme B mRNA analysis was performed twice and identical results were obtained. In terms of absolute values, granzyme B was expressed at very low levels compared to the other neutral proteases: for example, the expression of tryptase was, on average, $3 \times 10^6$-fold compared to that of granzyme B (data not shown).

### *5.3.6. Histidine decarboxylase*

The mRNAs for HDC were also detected at the first culture week and remained near this level also during the second culture week (Fig 6; *right panel*). After 3 weeks of culture an increase in the expression levels was observed, and beyond that time a relatively constant level of expression was achieved without decline in the expression by 9 weeks of culture when the analysis was discontinued. Compared to the neutral proteases, HDC was expressed at relatively constant levels between MCs of different donors.

### 5.4. Cellular histamine

Histamine measurement was initiated at 3 weeks of culture. At that time the amount of cellular histamine was, on average, 1 pg/cell (n=2 donors in duplicate experiments). The amount of histamine remained at this level up to 4 weeks of culture, and beyond that time increased along with culture being on average 8.5 pg/cell (n=2 donors in duplicate experiments) at the time of 9 weeks when the culture was discontinued (Fig 7).

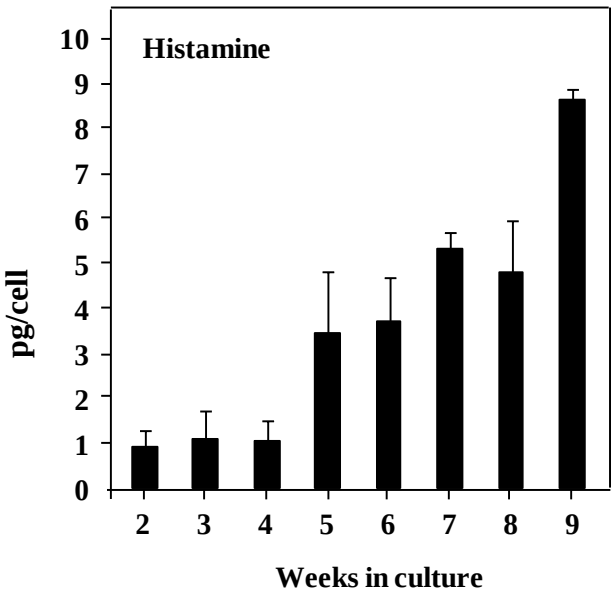


**Figure 7. Histamine content in developing MCs.** Data are means ± SD from at least two donors in duplicate experiments. SD; standard deviation.

### 5.5. Immunocytochemical stainings of mature mast cells

To compare the sensitivity of fluorescence microscopy to flow cytometry in detecting neutral protease expression, cytospins of cultured MCs at week 9 of culture were perfomed and stained with antibodies against human tryptase, chymase, cathepsin G, CPA3, and granzyme B. As shown in Figure 8, there was negligible autofluorescence or nonspecific binding in the samples based on isotype controls. Tryptase and cathepsin G stained the most intensively followed by chymase and CPA3. In contrast, granzyme B stained only weakly. Most importantly, some of the MCs appeared almost devoid of a particular protease (chymase and CPA3), even when flow cytometry clearly showed that all MCs were positive for all studies proteases at this time point. This observation addresses the critical dependence of the result on the detection method.

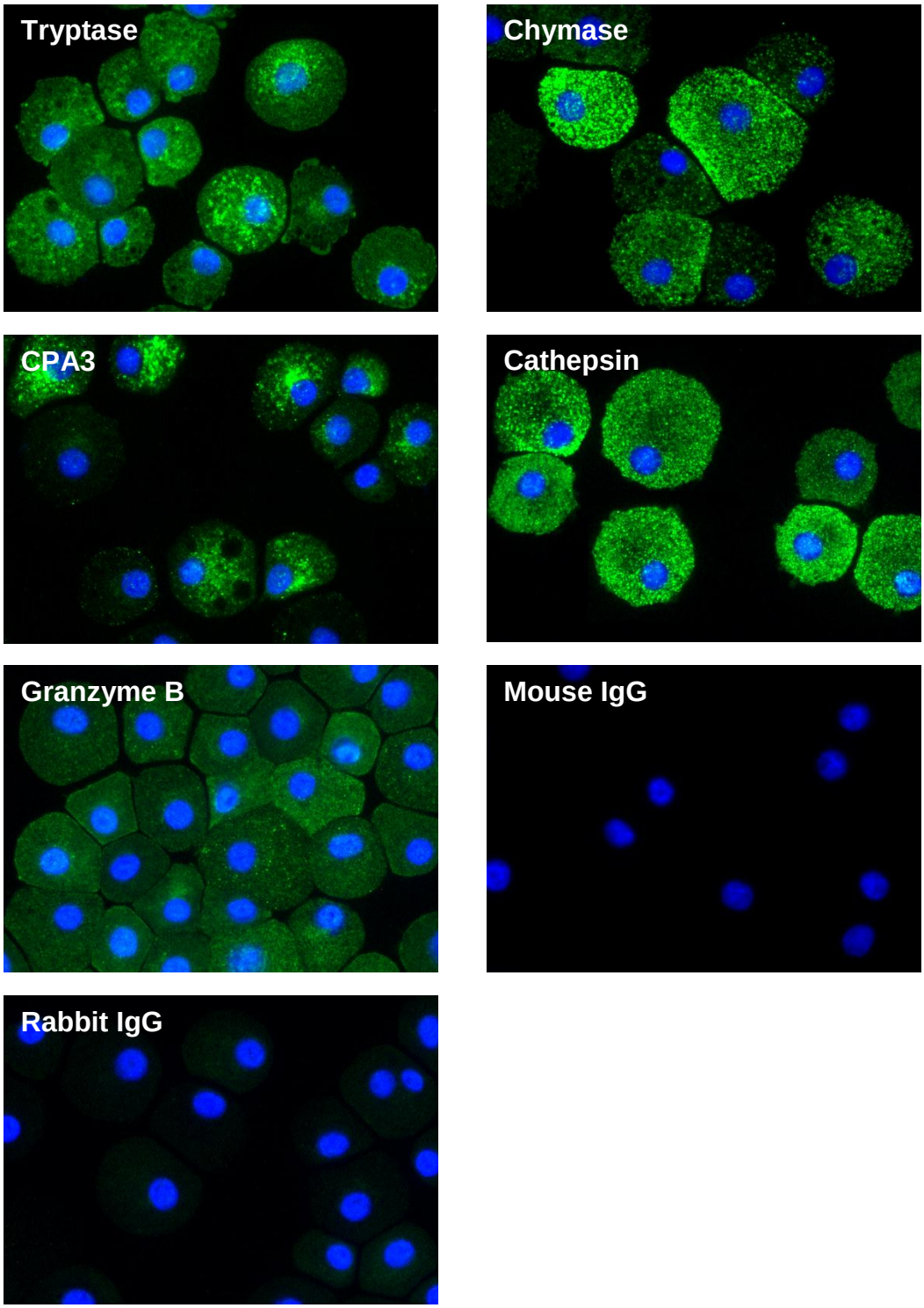


**Figure 8. Immunocytochemical stainings of human peripheral blood CD34$^+$ progenitor-derived mast cells at 9 weeks of culture.** Cells stained positively with mouse monoclonal antibodies against chymase and tryptase, and with rabbit polyclonal antibodies against cathepsin G, carboxypeptidase A3, and granzyme B. A fluorochrome-conjugated goat anti-mouse Alexa-488 IgG (for chymase and tryptase) or goat anti-rabbit Alexa-488 IgG (for cathepsin G, carboxypeptidase A3, and granzyme B) was used as the secondary antibody. Nuclei were stained with DAPI. Stainings with irrelevant IgGs (mouse IgG for tryptase and chymase, and rabbit IgG for cathepsin G, carboxypeptidase A3, and granzyme B) were used as negative controls.

# 6. DISCUSSION

The present thesis is partly based on a previously published study, which described a novel human MC phenotype, namely the tryptase$^+$, chymase$^+$, CPA3$^+$, cathepsin G$^+$, and granzyme B$^+$ MC (Maaninka et al., 2013). Before that study, four MC protease phenotypes had been reported within human tissues: 1) tryptase$^+$, chymase$^-$ MC ($MC_T$); 2) tryptase$^+$, chymase$^+$, CPA3$^+$, cathepsin G$^+$ MC ($MC_{TC}$); 3) chymase$^+$, tryptase$^-$ MC ($MC_C$); and 4) tryptase$^+$, CPA3$^+$ (Irani et al., 1986; Schechter et al., 1990; Irani et al., 1991; Weidner & Austen, 1993; Abonia et al., 2010; Dougherty et al., 2010). In human culture systems, the development of tryptase$^+$, chymase$^-$ MC ($MC_T$), tryptase$^+$, chymase$^+$ MC ($MC_{TC}$), and chymase$^+$, tryptase$^-$ MC ($MC_C$) had been demonstrated (Irani et al., 1992; Li et al., 1996; Toru et al., 1998; Kinoshita et al., 1999; Ahn et al., 2000; Shimizu et al., 2002; Lappalainen et al., 2007). Saito and coworkers had described cultured human MCs, which express transcripts for β-tryptase, chymase, CPA3, and cathepsin G (Saito et al., 2001), and granzyme B had been identified in cultured human MCs and in MCs isolated from human skin (Strik et al., 2007). Furthermore, after publication of the study, which this thesis is based on (Maaninka et al., 2013), yet another neutral protease, namely granzyme H have been identified within human MCs (Rönnberg et al., 2014).

Several protocols for *in vitro* differentiation of human MCs from their progenitors have been described (Irani et al., 1992; Kirshenbaum et al., 1992; Mitsui et al., 1993; Saito et al., 1996; Xia et al., 1997; Toru et al., 1998; Kinoshita et al., 1999; Ahn et al., 2000; Kambe et al., 2000; Kambe et al., 2001; Shimizu et al., 2002; Moon et al., 2003; Wang et al., 2006; Lappalainen et al., 2007). In such studies, MCs have been differentiated from their progenitors present in the bone marrow, fetal liver, cord blood, and peripheral blood under KITLG alone or in combination with T helper 2 (Th2) cytokines, such as IL-3, IL-4, IL-6, and IL-9. Differentiation under such conditions has yielded cultures comprising variable amounts of $MC_{TC}$ and $MC_T$ subtypes, and cultures consisting of MCs of the $MC_{TC}$ subtype exclusively. Irani and coworkers described the development of MCs from dispersed human fetal liver cells cultured with murine Swiss 3T3 fibroblasts (Irani et al., 1992). In these cultures $MC_T$ was the primary subtype observed. By day 30, there was no evidence of conversion of the $MC_T$ type into the $MC_{TC}$ type. Accordingly, the authors concluded that $MC_T$ and $MC_{TC}$ derive from two distinct progenitors with irreversibly predetermined protease phenotypes. Similarly, ultrastructural analysis of maturing human MCs in situ has led to conclusion that $MC_T$ and $MC_{TC}$ are committed subtypes (Craig et al., 1989).

The development *in vitro* of mainly $MC_T$ subtype in the presence of KITLG solely initially led to conclusion that KITLG alone might be insufficient to promote development of $MC_{TC}$ (Irani et al., 1992; Mitsui et al., 1993). However, Ahn and coworkers have demonstrated that human cord blood-derived MC progenitors develop into $MC_{TC}$ exclusively even when cultured under KITLG alone, an observation suggesting that all human MCs are capable of expressing chymase (Ahn et al., 2000). Ahn and coworkers also reported that chymase expression in cultured human MCs is clonally regulated at the progenitor level. Such cytokine-independent regulation of chymase expression could lead to apparent variation in chymase expression levels between MCs even in a single culture of $MC_{TC}$. The potential clonal regulation of other studied proteases is unclear; yet, MCs appear to express each neutral protease at a variable level, as indicated by the differences in fluorescence intensities observed in the present study by both flow cytometry and fluorescence microscopy. Indeed, light microscopy, including fluorescence microscopy, and flow cytometry have been widely used to detect MC neutral proteases stained with specific antibodies. Of these, flow cytometry is far more sensitive in detecting proteins, and was therefore used to detect MC neutral granule proteases in the present study. However, as a comparison, protease expression was also analyzed by immunofluorescence microscopy. As judged by the latter method, some of the MCs appeared devoid of chymase and CPA3, even when flow cytometry clearly showed that all MCs were positive for both neutral proteases. Such observation implies that the apparent protease-positivity of a MC may critically depend on the sensitivity of the detection method, which again may lead to variation in relative numbers of a particular MC subtype reported in any given sample based on the method used.

By reporting the development of a single tryptase$^+$, chymase$^+$, CPA3$^+$, cathepsin G$^+$, granzyme B$^+$ population of human MCs from their progenitors present in peripheral blood, the present Thesis suggests that all human MCs are capable of expressing a full set of the investigated neutral proteases. Given that KITLG alone is sufficient to induce tryptase and chymase expression in human MCs, it is intriguing to speculate KITLG as a common inducer of MC neutral protease expression in general. Indeed, this was confirmed by analyzing the expressions of tryptase, chymase, CPA3, cathepsin G, and granzyme B in MCs generated in the presence of KITLG alone (Maaninka et al., 2013). Binding of KITLG to KIT recruits downstream signaling molecules, including lineage-restricted factors, such as the basic helix-loop-helix leucine zipper transcription factor called microphthalmia transcription factor (MITF) (Phung et al., 2011). In humans, MITF-A is the major MITF isoform in MCs, and it regulates the transcription of βI-tryptase (Lee et al., 2010). However, in mice, MITF regulates the expression of several MC chymase and tryptase

genes, as well as the genes coding for cathepsin G and granzyme B (Pejler et al., 2007). Thus, MITF might be a key target of KITLG in the regulation of MC proteases in general. The recent study of Rönnberg and coworkers (Rönnberg et al., 2013) demonstrates that expression of granzyme D, the functional counterpart of human granzyme H, accompanies the KITLG-dependent differentiation of bone marrow precursors into mature bone marrow-derived MCs, supporting the hypothesis of KITLG as a common inducer of MC neutral proteases.

The data of the present Master's Thesis and the study, which this Thesis is based on (Maaninka et al., 2013) suggest that the expressions of tryptase, chymase, CPA3, cathepsin G, and granzyme B are an intrinsic feature of human MCs, and no distinct MC progenitors with irreversibly predetermined protease phenotypes exist. In mice, all MCs derive from a common progenitor cell, and the protease phenotype of MCs is highly dependent on cytokines and other factors present in the microenvironment surrounding the MCs (Kobayashi et al., 1986). In culture and *in vivo*, single CTMC may differentiate into an MMC, and vice versa (Sonoda et al., 1986; Kitamura et al., 1987). In addition, MCs with intermediate protease expression have been found (Friend et al., 1996; Friend et al., 1998). These observations suggest that mouse MCs retain the ability to reversibly alter their protease phenotype as a response to changes in their local microenvironment (Ghildyal et al., 1993; Friend et al., 1996; Friend et al., 1998). Changes in the protease phenotype have also been reported in human MCs. However, the extent, to which such changes are reversible in individual MCs, is not clear. *In vitro* studies have shown that human MCs, which contain tryptase but little or no chymase, may upregulate their chymase expression during incubation with IL-4 and IL-6, resulting in a change of the phenotype from $MC_T$ to $MC_{TC}$ (Toru et al., 1998; Kinoshita et al., 1999). Depending on the source of the progenitor cells such phenotypic switch may require an exceptionally long period of culture, even months, as described previously (Kinoshita et al., 1999). This could explain the contradictory result previously reported by Irani and coworkers (1992), who did not observe phenotypic switch of $MC_T$ to $MC_{TC}$ within 30 days. Besides *in vitro*, MCs are able to upregulate chymase also *in vivo*, as described in patients suffering from pulmonary vascular disease (Hamada et al., 1999). Human MCs may also switch their phenotype from $MC_{TC}$ to $MC_T$, as indicated by downregulation of chymase expression in $MC_{TC}$ cocultured with human airway epithelial cells (Hsieh et al., 2005).

The exposure of human cord blood-derived MCs to a conditioned medium derived from IL-13-activated epithelial cells resulted in the downregulation of chymase mRNA, while the mRNA expression levels of CPA3 and tryptase remained unaffected (Dougherty et al., 2010). In another

study describing gene expression in human tryptase$^+$, CPA3$^+$, chymase$^-$ MCs, CPA3 and tryptase mRNAs were upregulated in esophageal samples of patients with eosinophilic esophagitis compared with healthy controls, whereas no increase in chymase expression was observed (Abonia et al., 2010). These studies provide evidence that the expression of various MC neutral proteases may be individually regulated. This hypothesis is further supported by the recent study of granzyme B and H expressions in cultured human MCs and LAD2 cells. In these cells, activation by IgE and calcium ionophore resulted in upregulation of granzyme B expression, whereas granzyme H expression was downregulated, suggesting that these proteases are reciprocally regulated (Rönnberg et al., 2014). As the various neutral proteases have distinct substrate specificities, factors that can regulate the expression levels of these proteases in MCs will also potentially influence the extracellular functions that these cells have after secretion of the proteases.

The preservation of distinct types of MCs in multiple species implies unique and non-overlapping roles for these subtypes. The understanding of functional differences between MCs of various phenotypes is limited, however. According to one hypothesis, $MC_T$ play a proinflammatory role, whereas $MC_{TC}$ specialize in tissue remodeling (McNeil & Gotis-Graham, 2000). This hypothesis is supported by the promotion of $MC_T$ phenotype by T cells (Irani et al., 1987; Bentley et al., 1996), by the accumulation of $MC_T$ and $MC_{TC}$ to inflamed and fibrotic tissue areas, respectively (Gotis-Graham & McNeil, 1997), and by the preferential expression of the inflammation-associated protease tryptase by $MC_T$ and the extracellular matrix (ECM)–degrading proteases chymase and cathepsin G by $MC_{TC}$ (Leskinen et al., 2003; Mäyränpää et al., 2006; Kovanen, 2007; McNeil et al., 2007). However, not all observations fit into this dichotomy. For instance, tryptase has been reported to act on various ECM components or compounds related to ECM turnover (Pejler et al., 2007), and the potently proinflammatory anaphylatoxin receptor C5aR (CD88) is expressed on $MC_{TC}$, but not on $MC_T$ (Oskeritzian et al., 2005). At the moment, limited knowledge exists on the functional importance of the two major subtypes of human MCs, not to mention of other human MC subtypes with additional protease phenotypes, to permit firm conclusions. Analysis of the expressions of the whole variety of MC neutral proteases in MCs isolated from various tissues, both normal and diseased, would help clarify the unique roles of MCs with a particular phenotype.

# 7. CONCLUSION

Based on the experimental data of the present Thesis and the current literature, it is suggested that all human MCs derive from a common circulating progenitor cell with the potential to express the whole variety of human MC neutral proteases. Thus, the protease phenotype of a particular MC appears to represent a functional state the MC has assumed under the local microenvironment, and is subject to change along with the local microenvironment. Finally, better understanding of the factors that regulate MC protease phenotypes is required for a successful design of treatment strategies aimed at combatting MC-associated diseases, in which the various proteases released by activated MCs contribute to the development and progression of the disease-specific pathologies.

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